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CANINE PANCREATIC FRAGMENT TRANSPLANTATION: IMMUNOSUPPRESSION AND CRYOPRESERVATION

BY

ⓒ NORMAN MILES KNETEMAN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
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Abstract

Insulin therapy has been unable to control the severe chronic complications and premature death suffered by a large number of diabetic patients, especially those with insulin-dependent diabetes mellitus. Transplantation of pancreatic islets shows promise of providing optimal control of carbohydrate metabolism and so of preventing the debilitating chronic complicatins of insulin dependent diabetes mellitus.Successful models of pancreatic islet autotransplantation have been developed in large animal but several barriers to human islet transplantation remain: isolation of islets from human pancreata, suitable methods of storage to facilitate a large scale transplantation program, and methods to prevent destruction of the allografted islets by the immune mechanisims of the recipient. This study addresses the latter two problems.

Pancreatic dispersed fragments from excised canine pancreata were prepared by collagenase ductal perfusion, mechanical chopping, vigorous agitation and filtration. Control animals were left apancreatic or were immediately autotransplanted by slow injection of the islet containing fragments into the splenic vascular space by way of cannulated hilar veins. Three groups of animals received allotransplants and were either not immunosuppressed, or received azathioprine 3 mg/kg/day and prednisone 2 mg/kg/day or Cyclosporin A (CsA) 25 mg/kg/day. Storage of islets in liquid nitrogen was performed after slow cooling (0.25°C/min) to -75° after step equilibration with dimethyl sulfoxide (Me_2SO) to a 2 M concentration. Thawing at 3.5%C/min, Me_2SO removal with 0.75 M sucrose and step dilution preceeded autotransplantation. A final group received allotransplants of frozen-thawed tissue to evaluate cryopreservation as a method of ex-vivo immunological alteration. Evaluation consisted of plasma levels of glucose and insulin to monitor engraftment and subsequent rejection, monitoring for drug toxicity (hepatic, renal and hematologic function), as well as intravenous glucose tolerance testing (IV GTT) and weight change as indicators of metabolic control in acute and cryopreserved autografts.

Apancreatic controls died by 6.0±0.5 days. Four of 5 acutely autotransplanted animals maintained normoglycemia, and 3 of 5 nondiabetic IV GTT K values, for > 18 months (K=1.51±0.24). Glucose tolerance was reduced from preop values (3.26±0.13), as were peripheral plasma insulin levels following glucose infusion. Four of 4 dogs in the final group to receive frozen-thawed tissue maintained normoglycemia, and 3 of 4 nondiabetic K values, for > 12 months (K=1.56±0.26). After loss of 10 - 15% body weight in the first month post-transplant, animals of both groups were able to stabilize and then increase body weight.Non immunosuppressed allotransplanted dogs rejected by 5.0±0.6 days and died by 16.0±3.4 days; dogs treated with azathioprine and prednisone showed a decrease in duration of normoglycemia and survival but immunosuppression with Cyclosprin A delayed rejection to 19.3±5.6 days and increased survival to 33.3±4.6 days. Cyclosprin A was associated with no notable toxicity. Cryopreservation of islet containing tissue prior to allotransplantation did not prolong graft function.

Cyclosporin A 25mg/kg/day was able to delay rejection and prolong survival of allotransplanted dogs without causing notable toxicity; neither azathioprine-prednisone immunosuppression nor ex-vivo treatment by cryopreservation were able to achieve either. Cryopreservation was

able to store pancreatic fragments without deterioration of long term metabolic control after transplantation.

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I INTRODUCTION

It is estimated that there are 11 million diabetics in North America; 5 million diagnosed (a prevalence of 2.3% of the population) and 5-6 million undiagnosed. In 1964 325,000 new cases were diagnosed and in 1973, 612,000 cases (an incidence of 0.3%)(1). This may simply reflect better investigation of the population and better reporting but it is also likely that the true incidence of diabetes mellitus (DM) is increasing; by some estimates as much as 6% per year. D.M. is at present the fifth leading cause of death in North America with 15.2 deaths/year/100,000 population. In 1977; a diabetic's life expectancy was only 2/3 normal(2).

The devastating complications of diabetes are manifest in several main ways: acute hyperglycemia (with or without ketoacidosis) and coma, cardiovascular illness, renal failure, neuropathy, blindness and a host of metabolic and biochemical abnormalities. Evidence of coronary artery disease is increased tenfold in the adult diabetic population; and the incidence of myocardial infarction is doubled.(3) Severity is also increased; the post MI mortality is doubled in diabetics. Diabetics suffer 3-4 times more strokes than others and develop gangrene of the limbs five times as often. Ten years after being diagnosed as diabetic, between 5 and 52% of patients will have demonstrable peripheral neuropathy.

Chronic pyelonephritis occurs in 35% of diabetics, asymptomatic bacteria in 40% (vs 4-8% of the normal population). Diabetic glomerulosclerosis is present in 10% of diabetics; insulin dependent diabetes mellitus (IDDM) of over 10 years duration is associated with virtually a 100% prevalence of glomerulosclerosis, changes often being found within 5 years of diagnosis. Twenty percent of new hemiodialysis patients are diabetics and 3000 diabetics die from renal failure yearly in the U.S.A. Diabetes is now the second leading cause of renal failure in North America. Hemodialysis is a major difficulty in the diabetic with only a 60% 2 year survival reported in 1980. Transplantation is likewise affected with a 80-90% 2 year survival with living related donors and only 50-60% 2 year survival with cadaver donors. The changes of glomerulosclerosis may be seen in the transplanted kidney as early as 2-3 years after transplant(1).

Diabetics suffer an increased rate of catarract formation, twice the usual increase of glaucoma, and a 25 fold increase in blindness. Diabetic microangiopathy is observable in most cases within 10 years. Diabetes is the leading cause of new cases of blindness, 5000 yearly in the U.S.A.(1)

Why does diabetes, a disease thought to result mainly from defective biosynthesis, release and/or utilization of insulin, result in such widespread and serious long term complications, even when exogenous insulin administration is being used to prevent the lethal acute problems. The host of metabolic derangements in diabetes probably holds the answer.

A. non enzymatic glycosylation of proteins - ubiquitous but accelerated by hyperglycemia(1) Hyperglycemia seems to require the presence of other factors determining the susceptibility to glycosylation in order for microangiopathy to develop. Patients may have many years of poor diabetic control and yet avoid microangiopathy(15). Others develop changes of glycosylation in hemoglobin (HbA_{1c}), lens crystallins and glomerular basement membranes(15a)

B. hyperaggregable platelets: decreased fibrinolysis and decreased prostacyclin(12)

C. hyperviscosity of blood

D. circulating immune complexes which may cause basement membrane damage and thickening and microvascular changes(12).

E. impaired intracellular killing of bacteria by phagocytes(13).

F. elevated lipid and lipoprotein levels(14).

Evidence points to disorderd carbohydrate metabolism as being instrumental. Uncontrolled diabetics experience sustained hyperglycemia, standard depot insulin therapy helps to normalize plasma glucose to some degree but hyperglycemia and wide swings in glucose level are not eliminated.

Animal models of diabetes have shown a relationship between degree of metabolic control and the development of microangiopathy similar to human diabetes(4). Prospective human studies have also illustrated the parallel between plasma glucose control and onset and severity of complications(5-8). Identical complications have also followed non spontaneous diabetes induced in man by total pancreatectomy(9) or resulting from pancreatic destruction by hemochromatosis(10) and chronic pancreatitis(11). Intensive efforts to normalize the metabolic situation in D.M. are justified.

The most obvious method of maintaining rigid control of blood sugar is more frequent insulin administration - multiple subcutaneous insulin injections. In selected well motivated patients "near normoglycemia" has been achieved(17). Unfortunately a minority of diabetics are ideal candidates and complications include occasional worsening of retinopathy[16] and the possibility of hypoglycemic encephalopatly or death.

Mechanical systems to monitor blood glucose and use injections of either insulin or glucagon to regulate the sugar level, the "artificial pancreas", can achieve excellent control of blood glucose(18). These external "closed loop" systems, however, require continuous vascular access and entail relatively large amounts of blood loss for monitoring. Problems with infection, maintaining long-term vascular access and the considerable bulk of the equipment involved has restricted their use to experimental or short term in-hospital clinical use.

Portable "open loop" systems have been developed which dispense with blood sugar monitoring and rely on a programmed system of continuous basal insulin secretion with pulses at mealtime to achieve impressive control of blood sugar in many cases. Normalization of several metabolic abnormalities never corrected with depot insulin therapy have also been achieved including: serum lactate, pyruvate, free fatty acid, alanine, branched chain amino aid, plasma lipid and lipoprotein levels and glycosylated Hb(19). Documented improvement in peripheral nerve function was shown by others(20). Improvement in diabetic retinopathy and neuropathy have been much less dramatic(21). Complications at the site of subcutaneous injection include painful lumps and abscesses. Since no depot insulin is present, hyperglycemia would result rapidly from cessation of infusion.

By far the most serious complication, however, is hypoglycemia. Diabetics with autonomic neuropathy have a blunting of the signs of hypoglycemia and nocturnal hypoglycemia is expecially dangerous, severe encephalopathy or death may result. The type I (insulin dependent diabetes mellitus-IDDM) diabetic has been shown to be especially vulnerable to hypoglycemia since the normal glucagon response to hypoglycemia is generally absent in IDDM (23,23a,23b) and even the catecholomine based response to profound hypoglycemia may be lost(24).

The ideal means to not only sense and respond to hyperglycemia but to prevent hypoglycemia and normalize CHO metabolism is the pancreatic islet with functional alpha, beta, and delta cells complete with the complex interplay of controls between them and with their external milieu. The only method of achieving this goal in the patient with diabetes (esp. IDDM) is by transplantation of functionally normal pancreatic islets.

II REVIEW OF LITERATURE

A History of Diabetes Mellitus

The first known reference to diabetes mellitus was in the oldest known "text" of medicine, the Ebers papyrous (1500 B.C.). Celsus (30 B.C.- 50 A.D.) described the disease of polyuria without pain but with emaciation and prescribed a diet of as little food as possible. Aretaeus of Cappadokia (A.D. 30-90) named the disease diabetes (Greek for to run through a siphon), outlined its symptoms, its progressive nature and its ultimately fatal outcome(27).

Indian doctors in the 6th century A.D. noted the sweet taste and sticky touch of diabetic urine but it was not until 1674 that Thomas Willis rediscovered the idea in Europe and so developed the first useful diagnostic test to distinguish diabetes mellitus from other forms of polyuria. Willis was also the first in Europe to use dietary treatment by undernutrition. Matthew Dobson in 1766 observed that the sweetness of diabetic urine was due to sugar and Michael Chevreul in 1815 discovered the sugar to be glucose(28). Claude Bernard, the father of physiology, concluded that diabetes was a disturbance of normal nutrition, the function of the organism to compose and decompose chemical substances, but thought the problem lay in the liver(28).

It was not until 1889 that the pancreas was found responsible for preventing diabetes. Oscar Minkowski documented polyuria following total pancreatectomy in dogs(29). At the suggestion of Bernhard Nauyn (who subsequently suggested carbohydrate free diets and described lactic acidosis and its treatment with alkalis), Minkowski tested the urine and found it to contain glucose. Minkowski also noted polyphagia, hyperglycemia, ketosis with acetonemia, emaciation with loss of glycogen in organs, and documented poor wound healing and low resistance to infections in his dogs as in human diabetics. Furthermore, he showed that the results were not due to a loss of secretion into the GI tract but to a special, "still unknown", function of the gland. He was the first to attempt feedings of pancreatic extracts for the treatment of diabetes. Many others attempted injections of pancreatic extracts but the severe toxicity of the mixed endocrine and exocrine extract prevented success.

Paul Langerhans was the first to describe the pancreatic islets (1867). G.E. Laguesse named them the "islands of Langerhans" in 1893 and suggested that they might be the organ of pancreatic internal secretion. E.L. Opie and L.C. Sscobolew independently revealed the first clinical evidence linking diabetes mellitus to the islets: Opie by observing hyaline degeneration in the islets of diabetics at autopsy(28).

It was not until July 30, 1921, however, that F.G. Banting and C.H. Best, working in Dr. J.J.R. MacLeod's lab at the University of Toronto, succeeded in preparing a pancreatic extract from duct ligated glands that could control hyperglycemia in pancreatectomized dogs(30). They named the substance "Isletin" but changed the name to insulin on the insistence of Professor MacLeod.

The substance was purified chemically by J.B. Collip and January 11, 1922 saw the first use of insulin in a diabetic patient. Banting was also one of the first to realize that:

> "Insulin is not a cure for diabetes; it is a treatment."(31)

Pancreatic Transplantation

Surgical attempts to cure diabetes were born in 1894 when Williamson suggested transplanting animal pancreata to human diabetics. Sscobolew revived the idea in 1902 and in 1927 Gayet and Guillaume controlled blood glucose in a diabetic dog for 12 hours using a transplanted pancreas(32). The major causes of failure in these early efforts were vascular thrombosis, pancreatitis, and graft rejection.

The discovery of insulin and its subsequent modification by Hagedorn (protamine insulin) and Scott (protamine zinc insulin) to provide prolonged duration of action shifted attention away from pancreatic transplantation for many years. Insulin provided a new life for diabetics by controlling metabolism well enough to allow normal functioning in society for many years. With this prolonged survival, however, came the realization that diabetes was also associated with devastating chronic complications that were not prevented by depot insulin therapy. A landmark in this series of revelations was the description of diabetic glomerulosclerosis by Kimmelstiel and Wilson(33). These findings led to a renewed interest in alternative treatment modalities in diabetes including pancreatic transplantation.

The rationale for attempts at transplantation of the pancreas is the idea of "total endocrine replacement" for IDDM, a phrase coined by Dr. D.E.R. Sutherland of Minnesota. Disordered carbohydrate metabolism, mainly sustained hyperglycemia, has been shown to result in the many chronic complications of diabetes. Presumbably, perfect control of carbohydrate metabolism could reverse these changes or at least halt their progression in established diabetics and prevent their development in new cases.

Animal models have lent support to the hope that pancreatic transplantation could control or even reverse the complications of diabetes. Renal lesions, similar to those seen in human diabetes, develop in experimentally induced diabetes in animals. Pancreatic islet transplantation in diabetic rats has resulted in a decrease in mesangial matrix material(34,35) as well as halting the progressive increase in basement membrane thickness(36), and a rewidening of the capillary lumen(37). Both islet transplantation(38) and whole organ transplantation(39) prevented neovascularity in rat eyes and even resulted in regression of eye pathology in diabetic rats(40). Other effects, including those on diabetic neuropathy, have been documented by others.

Whole Organ and Segmental Transplantation

A new era in the therapy of diabetes began in December 1966, when Kelly, Lillehei and associates performed the first human pancreas transplant: a combined renal and segmental pancreatic transplant(41). The duct ligated gland was placed retroperitoneally in the iliac fossa and allowed insulin withdrawal for 6 days; unfortunately, the pancreas and kidney were removed 2 months later and the patient succumbed to venous complications of the grafts.

Largiader reported a successful orthotopic allotransplant of the canine pancreas in 1967(42). He utilized cuffs of donor coeliac artery and portal vein to facilitate the vascular anastomosis and drained the pancreatic duct by sewing a retained cuff of duodenum to a Roux-en-Y jejunal limb. Lillehei and associates adopted these ideas and reported ten pancreaticoduodenal grafts in 1970(43). Almost all functioned at least briefly but there was no long-term successes and no survivors over 1 year. A major cause of failure unique to this approach was necrosis and rejection at the duodenojejunal anastomosis.

These poor results led to concentration on segmental pancreatic grafts. Problems with graft vessel thrombosis were decreased by the simple two-vessel revascularization; no bowel was attached so rejection was easier to control and the possibility of eliminating any opening of the GI tract lessened the chance of graft pancreatitis due to activation of exocrine enzymes by intestinal content. The major problems still to be resolved were the disposal of ductal secretions and the control of rejection.

Anastomosis of the pancreatic duct to the divided ureter in uremic patients was attempted by Gliedman with some success(44) but this approach was obviously restricted to patients without renal function. Duct-ligated models had been successful experimentally but clinical application was not so due to leakage of enzyme rich fluid out through lymphatics to form perigraft collections, as well as the severe inflammatory rection in the gland that followed and the conflicting reports of its damaging effects on the islets(45).

This approach is now used in the majority of European centres, who feel that the safety factor achieved by avoiding a GI anastomosis (with the possibility of enzyme activation) is a fair trade off for the possibility of loss of endocrine function subsequent to the fibrotic reaction stimulated by the injection. Leakage of enzymes from residual acinar tissue remains a major problem with duct injected models with fistulas forming in 35% of patients on the Lyon series (causing death in 1 of 19 leaks)(46) and 40% of the Munich series (with 10% of grafts lost due to the septic focus(47).

Kyriakides demonstrated in 1979 that non ligated glands could be transplanted to the peritoneal cavity in pigs without problem(48). Clinical usage was not without problem, however, as the capacity of the peritoneal cavity to absorb the exocrine secretion may be insufficient, resulting in intractable ascites. All centres have now abonded this approach.

Dubernard developed a technique to obliterate the ductal system by Neoprene injections(49) and numerous other substances have been used including acrylate glue, polyisoprene, silastic and prolamine.

Because of the possibility of deterioration of islet function in the long term with the duct injected model, nearly all American and British groups have again began utilizing drainage of the ductal system. To avoid the difficulties with enzyme activation inherent in enteric drainage, Munda and Alexander in Cincinatti have resurrected the technique of drainage to the ureter(50) and Sollinger and Belzer in Madison utilized a technique of drainage into the bladder(51). Early results of both groups are promising. The Minneapolis group now utilize enteric drainage exclusively, with segmental transplants in their cases of living related donors (now 40% of their transplants). Because of the marginal metabolic control achieved by some segmental grafts, the Minneapolis group now prefers total pancreas transplantation in all cases of cadaver donors(52). They believe the increased islet cell mass transplanted also provides a worthwhile buffer against rejection.

The second major cause of technical graft failure has been vascular (usually splenic artery) thrombosis. Traeger and Dubernard at Lyon have lost 16% of all their grafts to thrombosis(46) and Land's group at

Munich have lost 7% due to thrombosis despite fully heparinizing their patients(47). The commonest method used (besides heparinization) to prevent thrombosis has been construction of an AV fistula distally between splenic artery and vein. Thrombosis has been seen despite AV fistula formation in both human cases and animal models however(50,53). The Cincinatti group now transplants the whole pancreas together with the spleen to normalize the vascular flow as much as possible(50). The immunologic consequences of transplanting the spleen could well be substantial, including the possibility of introducing graft versus host disease.

Despite the tremendous amount of experimental work and the sizeable clinical experience with 485 human pancreas transplants reported, problems still abound. Graft vessel thrombosis still occurs, as does graft pancreatitis and the ideal solution to ductal handling remains to be established. Despite the fact that these grafts have been shown to be metabolically efficient (54,55), the numerous technical problems, combined with allograft rejection, have resulted in infrequent long-term success with either whole organ or segmental transplants.

In the 425 transplants done worldwide since 1977 (40 from living related donors), 1 year patient survival has been 75% and 1 year actuarial graft survival 29%(56). Thus only 24% of patients have survived 1 year with graft function intact. The University of Minnesota group remains the most experienced in the world; their results for the last year still show only 30% graft survival for 1 year when cadaver donors are utilized(52).

Results with pancreatic transplantation have improved, however, and because of the promise of improved metabolic control and decreased complications in insulin dependent diabetics, the number of centres performing pancreatic transplants and the total number being done is mushrooming. Early evidence is being seen of the ability of total endocrine replacement by transplantation to reverse the complications of diabetes. Land has demonstrated improved nerve conduction velocity in 4 of 5 long term graft successes studied(47). The Minnesota group has documented reversal of early diabetic nephropathy in transplanted kidneys in 2 patients with pancreatic grafts functioning for over 4 years, as well as regression of preproliferative retinopathy in 3 patients(52). Nevertheless, pancreatic transplantation continues to have a substantial rate of both morbidity and mortality, and the results have never been subjected to a controlled trial in human recipients.

Pancreatic Islet Transplantation

An alternative approach to achieving "total endocrine replacement therapy" for diabetes mellitus is to transplant only the pancreatic islet, after separation from acinar tissue, as a free endocrine graft. This approach offers many theoretical advantages. No vascular anastomoses are necessary so complications of hemorrhage and graft vessel thrombosis are eliminated. Graft pancreatitis occurring in the tiny dispersed particles would not be a major threat and the problems with exocrine secretions would be eliminated. The grave problems of infection in the graft would be minimized and there would be no need to remove failed grafts. The possibility also exists for multiple transplants from a single donor gland. This has been accomplished in the rat by Payne *et al.*(57).

It was initially throught that islet cells would be, like ovary, thyroid and many other endocrine tissued, only weakly immunogenic(58), and so elimination of exocrine tissue was hoped to alleviate problems with rejection. Unfortunately, most experience with dispersed islet tissue grafts has seemed to indicate they are at least as immunogenic as whole organs(59,60) and to be exquisitely sensitive to the effector mechanisms of rejection, both cellular and humoral(61,62,63).

Successful transplantation of isolated pancreatic islets requires all the factors outlined for free grafting of other endocrine tissue:(64)

(A) The method of preparation must conserve a critical mass of tissue.

(B) Tissue fragments must be of appropriate size to survive by nutrient diffusion until neovascularization occurs

(C) The site of implantation must be appropriate.

Pancreatic islet transplantation requires some unique considerations as well:

(D) A method to achieve isolation of the islets from the surrounding exocrine tissue.

(E) How to protect the graft and the host from the destructive effects of the proteolytic enzymes secreted by residual exocrine tissue.

(F) How to maximize the physiological effects of the hormones released from the islets.

A review of past efforts in pancreatic islet transplantation can outline the successes and failures overcoming the above problems. The subsequent section will serve as a background to current efforts.

Ivy and Farrell were the first to attempt non vascularized pancreatic transplants (58). They noted deterioration of exocrine function and continued functioning of islet tissue in the abdominal wall. W.A. Selle was the first to attempt transplantation of pancreatic fragments (65). He tissue cultured and then homotransplanted 1-2 mm fragments subcutaneously in dogs, but with no evidence of function. Brooks used the technique of Ivy and Farrell to successfully autograft pancreas (66). A major advance occurred in 1965 when Moskalewski utilized collagenase, an enzymatic complex produced by bacteria of the genus Clostridia, to separate intact functional islets from chopped guinea pig pancreas (67).

Lacy and Kostianovsky improved collagenase digestion by mechanically disrupting the pancreas with an injection of salt solution down the common bile duct, into which the pancreatic ducts drain (68). They also applied a method of discontinuous density gradient separation using sucrose concentration gradients and centrifugation to allow more efficient separation of intact islets from acinar tissue. This method allowed separation of relatively large numbers of islets but the hyperosmolar sucrose damaged the islets resulting in poor insulin release. Ficoll, a high mollecular weight (mw=400,000) nonionic synthetic polymer of sucrose (69) which is less toxic to islets if dialysed prior to use, was later used in varying concentrations to achieve comparable islet yields with normal islet function (70).

In 1972, Ballinger & Lacy transplanted 400 to 600 isogeneic islets (that had been obtained from 4 rats) into the peritoneal cavity or intramuscularly in rats that had been previously made diabetic by injection of the β cell toxin Streptozotocin. Although normoglycemia was not routinely obtained, the animals survived, and gained weight and had lower fasting blood glucose levels and less glycosuria than control animals. Rats with allogeneic islets treated with Azathioprine 4 mg/kg/day faired nearly as well. This was the first demonstration of long term amelioration of experimental diabetes by isolated adult islets (71).

A major problem with these methods producing isolated intact islets was the low percentage of functional islet tissue they could recover. Continued use of multiple donors would create formidable problems with rejection of allografts and low yields prevented application of these techniques to large animal models. Mirkovitch and Campiche surmounted these difficulties in a canine model by eliminating attempts to separate exocrine and endocrine tissue. Using intrasplenic injection of unpurified particles of pancreas up to 2 mm in diameter, obtained by simple chopping and collagenase digestion, they produced sustained post operative normoglycemia for the first time in a large animal model using a single donor pancreas(72).

This landmark work by Mirkovitch and Campiche aroused a great deal of interest in pancreatic "dispersed fragment" transplantation. Between 1970 and December 1981, 76 islet allotransplants performed in 71 diabetics were reported to the International Transplant Registry (73). Most used variations of Mirkovitch's method and nearly all failed. Only 4 patients were reported to be insulin independent for prolonged periods and only one of these was well documented. Largiader et al. in Zurich performed a simultaneous renal and intrasplenic islet allotransplant from a 2 1/2 year old donor to a 33 year old woman who had been a severe diabetic for 22 years (74). Postoperatively the patient required insulin but despite 3 severe rejection episodes she became totally insulin independent 8 months post transplant and remained so until 20 months post transplant when she simultaneously rejected her kidney and became hyperglycemic.

Despite the large proportion of failures,these occasional successes were encouraging. Perhaps if problems with rejection could be overcome, islet transplantation would be possible. A situation seemingly designed to test this possibility was readily available: patients undergoing total pancreatectomy for chronic pancreatitis became severe, brittle diabetics. Najarian, et al., in Minneapolis, succeeded in achieving insulin independence in 3 of 10 patients who underwent over 95% pancreatectomy by an intraportal autotransplant of unpurified collagenase digested pancreatic fragments (75). Cameron et al. , in Baltimore, achieved insulin independence in 3 of 12 patients after 95% pancreatectomy and documented insulin production from the intrahepatic transplant site in one (76). In total, 34 of 73 cases have been independent of insulin for variable periods of time (73). Unfortunately only 2 of these had total pancreatectomies and only one of the others had documentation of a successful graft, i.e. insulin present in hepatic, but not portal veins(76).

Why have not more of these autotransplants been successful? Certainly a major difficulty, still, was obtaining a sufficient yield of islet tissue. Kretschmer, et. al., utilizing Mirkovitch's method of islet isolation in dogs obtained an average yield of only 8% of total islet tissue present in the gland prior to digestion.(77) The fibrotic nature of these glands makes digestion all the more difficult and the complications of intraportal pancreatic tissue injections compound the difficulties. The even poorer results with human islet cell allotransplants,however, suggests that immunological barriers remain the greatest obstacle. A review of the many problems still preventing successful human islet transplantation is now in order. Consideration of the many approaches to these problems and the results of these attempts will help define the picture more clearly and suggest alternative avenues of study, some of which we started down this year.

The success of an attempt to cure diabetes with an allograft of pancreatic islets depends on several major factors: A critical mass of viable islets must be obtained by the isolation procedure and they must be in an optimum form for engraftment in the host. They must be successfully stored until the donor is ready for transplantation and then successfully placed in the site where engraftment most easily occurs and their hormonal products will exert maximal effect. Finally, a system for preventing the host from rejecting the graft must be applied, probably for life.

Islet Yield

Initial methods, successful in rodents, of hand picked islets from collagenase digested pancreatic tissue, or by obtaining islets by centrifugation on density gradients have not been practical in large animal models or humans since the pancreas in these species is more fibrous and at least a hundred thousand islets are necesary to maintain carbohydrate metabolism in humans. Applications of Mirkovitch's method have resulted in tremendous variability of yield (<5 to >50 percent of initial β cell mass) and an average in most series of only 5-10%(77). Methods to increase yield must not do so at the expense of viability of the resulting islets. Downing and Scharp achieved a four fold increase in islet yield by disrupting the canine pancreas with venous injection prior to collagenase digestion. They cite the islet-acinar portal venous system as the reason for more intralobular disruption with this method(78). Unfortunately, the islets did not maintain a normal insulin response to high glucose solutions during perifusion, signifying more severe damage to the β cells from this approach. The effect may only be temporary, however, as 24 hours in tissue culture restored the insulin secretion response.

Horaguchi and Merrell utilized perfusion of the pancreatic ductal system with collagenase solution followed by mechanical mincing and filtration through a 400μ screen(79). They achieved a 57% recovery of β cell mass (reflected by insulin recovery from graft vs. raw pancreatic tissue), a six fold purification of β-cell content, found 90% of the cells to be viable by trypan blue exclusion, and achieved post-operative normoglycemia in 3 of 5 dogs transplanted intraportally and 2 of 2 transplanted into the spleen. Using a modification of this method Warnock, Rajotte, and Procyshyn achieved a 24% β cell recovery and two fold β cell purification. Following transplantation using splenic venous reflux, 9 of 13 dogs became normoglycemic by the fifth post-operative day. Glucose tolerance tests on the surviving dogs produced K values of 1.4±0.1%, in the nondiabetic range, compared to 3.4±0.2% pre-operatively(80). The K value, or percentage decline in blood glucose per minute following an intravenous glucose bolus, assesses the functional ability of the β cells, in conjunction with other metabolic systems, to control carbohydrate metabolism.

This method seems to satisfy the needs for not only adequate yield of islets, but also viability before transplantation and satisfactory engraftment in the host, as evidenced by the ability to maintain carbohydrate metabolism in a nondiabetic range. The major influences affecting cellular viability in this method of preparation are warm ischemia and duration of exposure to the destructive actions of collagenase.

Problems stemming from the use of collagenase go beyond its toxicity. There is as yet no satisfactory in vitro method of assaying the activity of the enzyme. It is in effect an unproven biological product that is offered for sale. The standard industry assay of units per milligram refers to the micromoles of l-leucine liberated per milligram of enzyme in the mixture; unfortunately, this activity has not been found to correlate wih in vivo effects(81). Therefore, the published results in both small and large animal studies of islet transplantation utilizing collagenase digestion, depend to a substantial degree on the variable activity of the lots of collagenase used, and the supplier. The only way to assure efficiency of digestion without excessive toxicity from prolonged exposure, from a batch and lot of collagenase is a controlled trial in the method to be utilized in a given study(82), a very time consuming and expensive proposition (approximately $3,000 in time, materials, animals and operating costs in this study).

In an attempt to avoid the inconsistencies and the toxicity inherent in collagenase digestion, Hinshaw, et al. developed a totally mechanical method of isolating islets. Pancreatic tissue is hand minced, then pressed through a 280 µ seive. A low speed centrifugation and removal of the pellet reportedly eliminates most exocrine, vascular, and ductal contaminants before a final high speed spin settles the islets from the suspension to be saved for transplanting. While large islets (>280 µm) and islets not liberated from exocrine tissue would obviously be lost within this technique, hemocytometer counting has yielded estimates of 500,000 to 2,000,000 islets and islet fragments. Application to humans with chronic pancreatitis have achieved results similar to other methods (2 of 5 insulin independent following 95% pancreatectomy) without systemic heparinization and without significant rises in portal pressure following transplantation(83). Insulin production from the graft rather than the residual pancreas has not been established, however.

Islet Engraftment

Isolating adequate numbers of viable islets does not guarantee success in islet transplantation; a high rate of tissue engraftment must be achieved in the recipient. Two major factors are involved in successful engraftment:

1. The degree of tissue dispersion must be adequate(75)
2. The optimum site must be selected.

Tissue dispersion (particle size) must be sufficient to allow placement of the graft without excessive morbidity in the host due to physical size. Part of the difficulty with intraportal injection of islets preparations stems from simple mechanical obstruction of portal circulation contributing to severe portal hypertension or even stasis and thrombosis(84). More importantly the tissue particles must be small enough to survive by nutrient diffusion until neovascularization of the islets can occur in the graft site. Mehigan and associates have demonstrated a definite relationship between particle size and eventual outcome of pancreatic fragment transplantation(82).

The size of pancreatic fragments obtained for transplantion is dependent on several factors. The type and lot of collagenase and the time of exposure are influences that have been outlined by Mehigan *et al.*(82) and Kretschmer *et al.* (85) respectively. The fibrous nature of the pancreas also influences the ability to obtain viable fragments of a size and number sufficient to engraft and reverse diabetes. This will vary both with species involved and the age of the animal. The adult human gland is very compact and fibrous and so holds a great challenge. Compounding pancreatitis with further increase in fibrous tissue content may well aggravage the difficulties. Mehigan *et al.* demonstrated a poorer result in dogs with a duct ligation model of chronic pancreatitis using techniques that were successful in other dogs(86).

Subsequent digestion with trypsin(87) or dispase(89) or other enzymes can yield small fragments of islets or even single cell preparations. These smaller fragments should be more easily sustained by nutrient diffusion and thus would be preferable if they can be obtained without excess cellular toxicity from prolonged enzyme contact. Unfortunately, trypsin digestion has been shown to decrease the response of islets to stimulus with both glucose and tolbutamide, perhaps due to a damaging effect on the glucoreceptor mechanism on the cell surface(88). It has been suggested that tissue culture after trypsin digestion may restore normal response to a glucose load; it seems the derangement may be reversible.

Site of transplantation also influences the degree of engraftment. A large blood flow would help sustain viable cells by nutrient diffusion until engraftment could occur. In the rodent, islets have been successfully transplanted in many sites including subcutaneously, intramuscularly, in the pleural space, in the testis, subcapsularly in the kidney, intraperitoneally, in the spleen, and intraportally into the liver. These sites have not proven to be of equal benefit.

Transplant Site-Metabolic Effects

Optimal transplantation requires the grafted islets to be in a position where the excreted insulin and other hormones will have their maximum metabolic impact. Kemp et al. demonstrated the superiority of a site draining into the portal circulation in rats(89), presumably the result of the liver being the major site of carbohydrate homeostasis in mammals, and hence the major site of insulin action. Many intraportal sites have been utilized. Direct intraportal innoculation to the liver is the most effective method in the rat(89). This creates major problems in canine models because of the extreme sensitivity of the dog to intraportal pancreatic fragment injection with resultant portal hypertension and frequently death(90). This problem is not so severe in man but portal hypertension is still a problem and 2 deaths have been documented from intraportal injection in man because of severe prolonged portal hypertension compromising bowel viablility(91,92). Traverso and group have ameliorated this problem by adding heparin and aprotinin to the infusate, they believe the portal hypertension is caused by activated kinins in the infusate which may be bound and inactivated by aprotinin(93).

An alternative that has proven successful in both dog and man is a splenic transplant site. Again the requirements of high blood flow for nutrition and portal drainage for maximal insulin effect are met.

Kretschmer found direct innoculation of the islets into the splenic pulp to be the best method of transplantation(77) but Warnock _et al_. showed a deterioration in function in dogs transplanted by this method. In contrast, animals transplanted by splenic venous reflux maintained normoglycemia and a nondiabetic GTT for prolonged periods(80). The spleen is relatively simple to mobilize for surgical manipulation in the dog but is not an easy organ to work with in man.

In an attempt to avoid utilizing the spleen, Warnock _et al_. attempted embolization of pancreatic dispersed fragments into the stomach but the vasospastic content caused intense vascular spasm and eventual necrosis(94). Injection into the mesentery of the small bowel has been successful in the short-term, but fibrosis has compromised long-term function(95). Scharp has had some initial success with transplantion into a nitex lined omental pouch(95), but subsequent studies have not developed a reliable model with long term success.

Engrafted pancreatic tissue, in order to function for prolonged periods, must be protected from destruction by the exocrine enzymes secreted by the acinar tissue component. Hand-picked clean islets or pseudo islets formed from purified endocrine cell populations would not be subject to this poblem but at present no satisfactory method for these preparations exists in large animal models. Whole gland or segmental transplants avoid damage by the maintenance of the normal anatomic separation of islets from acinar tissues and their enzyme production; unfortunately the enzyme products cause other major problems which have been alluded to.

Transplantation of non-purified islets or pancreatic dispersed fragment preparations must allow for exocrine enzyme secretion. Early attempts at transplantation to subcutaneous and intramuscular sites failed partly because of extensive autolysis by these enzymes. The use of the liver and spleen as transplant sites has solved this problem to varying degrees. Some investigators have found evidence of fibrosis surrounding islet tissue in these sites, presumably due to tissue reaction to irritating cellular products. This may be the explanation for the limited life of islets transplanted to the spleen by direct innoculation of the pulp.

In contrast, islet tissue transplanted to the liver has been shown to engraft, pass the vascular endothelium and come to rest adjacent to parenchymal cells(89) without evidence of damage from exocrine tissue. Warnock et al showed a similar occurrence in the spleen by venous reflux of a dispersed fragment preparation(94). Initially, particles containing both exocrine and endocrine cells were engrafted in the splenic sinusoids. The cells eventually made their way across the vessel wall to the splenic parenchyma and the spleens of long-term animals showed a progressive decrease in amount of exocrine tissue. The critical factor in these two sites again appears to be a large blood flow, apparently necessary to "flush away" any toxic exocrine enzymes before they can reach dangerous levels or be activated.

While placement of transplanted islets into the portal system has been shown to maximize metabolic impact of the hormones released, it also causes problems, especially with accurate follow-up. Serum levels of insulin are unreliable indicators of total graft function in this situation. The largest source of utilization of insulin is the liver, an organ that is capable of removing and metabolizing much of the insulin content of the blood supplied to it in a single pass through the hepatic parenchyma.

Assays of portal or splenic vein insulin when combined with blood flow measurements can more closely monitor the hormonal production of engrafted islets but reliable vascular access to the portal system requires laparotomy for catheter placement. One alternative is the utilization of an assay for the C-terminal peptide group of proinsulin that is cleaved off prior to activation. C-peptide is not metabolized in the liver but is excreted unchanged by the kidney(96). Total urine content of C-peptide can therefore reflect activity of engrafted islets and peripheral venous content of C-peptide may be able to reflect dynamic changes in graft action.

Islet Sources

Clinical pancreatic transplantation on a scale that will provide help to many of the 2 million insulin dependent diabetics in North America will obviously require a large pool of donor organs. Several possible origins exist, only 2 being utilized at present.

The ideal donor is an HLA identical sibling, failing that, a living related donor is superior to a cadaver donor matched for an equal number of HLA haplotypes. Living related donors are currently being utilized in whole organ transplantation(52) but a mass program involving this type of donor is not practical, nor should it be pursued if alternative satisfactory sources can be found.

Cadaver donors provide the bulk of organs for renal transplantation and whole organ transplantation and will quite probably do likewise for clinical islet transplantion. Methods of preservation, isolation and transplantation in large animal models have largely involved young adult animals, giving this donor pool an advantage at present over other alternatives. Limited numbers, however, will probably continue to be a problem with cadaver donors.

Neonatal pancreas has definite advantages over adult tissue for transplant. Leonard found neonatal rat pancreas to contain about 5 times the insulin content of the adult gland on a per weight basis and a much lower exocrine content, indeed while adult pancreas is only about 0.5% islet tissue neonatal pancreas is nearly 5% islet mass(97). Because of this, neonatal tissue can be transplanted with much less preparation and so a higher proportion of islet tissue is ultimately available for transplant with current techniques. Despite this, even in rats, the small total amount of endocrine tissue in the neonatal pancreas has been only partially successful in reversing diabetes with single donor islet preparations transplanted(98). The supply of neonatal human tissue will obviously be severely limited.

Fetal pancreas also has high islet and low exocrine content, with almost no exocrine content despite well developed islets at early stages of development (about 17 days in the rat)(99) Fetal pancreas can also be transplanted without dispersal as a free organ graft by virtue of its small size(100). Unfortunately, unless complex transfers with intermediate hosts are used, it has not been possible to reverse diabetes unless multiple (about 4) donor glands were used. The ability of the fetal tissues to undergo proliferation minimizes the number of donor glands necessary but also results in a long lag phase prior to sufficient development to control diabetes; weeks to months in rat models(98). Fetal tissue is one of the few sources where supply could be plentiful, ethical and moral problems with utilization of tissue from aborted fetuses will certainly be a major problem, however. It was initially believed that fetal pancreatic tissue would be less immunogenic than adult tissue. Investigation has revealed little difference in the time for rejection to occur in adult or fetal rat islet transplantation(101). Indeed the high lymphoid tissue content of fetal pancreases could be expected to cause a greater immunologic response to transplanted tissue.

The ideal source for islet tissue to transplant would be animal xenografts. Supply would be limitless; moral problems minimized. The difficulties with rejection, however, would be of an order of magnitude greater than with allografted human tissues, and indeed, may not be solvable in the forseeable future. It now seems appropriate to turn our attention to this problem of the immune rejection process, one of the main stumbling block still preventing a mass program of pancreatic islet transplantation in our huge diabetic population.

III THE PREVENTION OF REJECTION

The process of immune attack on engrafted tissue is complex and still incompletely understood. The initiation of rejection occurs by sensitization of the host to antigen from the donor tissue which is recognized as foreign. However, current beliefs unlike early theories, hold that the mere presence of foreign antigens does not necessarily result in rejection (102). The antigen must be suitably "processed". A simplified outline of the proposed process follows.

Sensitization can occur in three ways.(103) Firstly, antigens released from the engrafted organ can enter the blood or lymphatic systems and be picked up by immunocompetent cells. Secondly, "passenger leucocytes", donor white blood cells transplanted in the graft, can pass via lymphatics to draining lymph nodes and incite a response at that site. Thirdly, host leucocytes in passing through the graft after engraftment has occurred may be exposed to the grafts "foreign" antigens and sensitized.

Foreign antigen is then thought to be "processed" by macrophages (free or tissue bound dendritic cells) and presented to T lymphocyte "helper cells". If the helper cell is able to recognize the antigen it can then interact with other T cells (cytotoxic or "killer" T cells) to initiate cell mediated immune attack on the foreign tissue or it can interact with B cells to initiate humoral immune responses. If preformed antibody to the foreign tissue is present in the host due to prior exposure to like antigen, hyperacute rejection may result. In other cases where no immunosuppression is present the cell mediated response usually predominates and the process known as acute rejection occurs. If acute rejection is suppressed by certain therapies, a largely humoral attack can still occur, mainly on the vascular supply to the foreign tissue resulting in chronic rejection.(103)

Several approaches may be followed to prevent the rejection response. Tissues may be "matched" by serological or cellular methods to minimize the histoincompatability between donor and host tissues and so minimize the immune response. Another approach to preventing the immune response or at least to prevent it from destroying the engrafted tissues is to sequester the graft within an "immunoisolation device" that will allow molecular exchange between the graft and host, and so physiological function of the graft, but will prevent cellular contact between the graft and host, thus preventing cell mediated immune destruction. A recent and exciting approach to preventing the immune response is the ex vivo immunological manipulation of the graft to minimize the stimulus to immune destruction. Finally, immunosuppressive therapy may be used in selective or non selective ways to prevent the full development of the rejection response and the destruction of the engrafted tissue.

Tissue Typing - Minimizing Histoincompatability

The 1950's saw the discovery of the HLA or histocompatability locus antigens. The thought of transplantation antigen as the major barrier to transplanting foreign tissues gave rise to the idea of matching donor and recipient tissues for these antigens to prevent the immune response from rejecting the grafted tissues. The first such antigens known were those associated with the HLA-A and B regions. Initial results from matching at these loci were hopeful and were enthusiastically accepted, long-term studies with critical follow up were not so dramatic however.

HLA-A, B and C are all serologically defined regions and are present on almost all tissues(104). The discovery of another region of the major histocompatability complex, HLA-D, that was defined not serologically, but initally by lymphocytic reaction in culture (mixed lymphocyte reaction), opened another door. Matching at the D region proved more successful than the other HLA loci and indeed seems to be as important as the other known HLA regions combined, the effect being additive to the benefits of matching the other loci(105). Further work gave clues as to the reason for this success.

Not every antigen was found to cause an immune response. Indeed it now seems likely that T lymphocytes and other immune cells can only "see" extrinsic antigens in the context of, or in association with, self major histocompatability locus antigens(106,107). For helper T cells and macrophages, the self antigens involved are the "Ia" antigens, coded for in mice by genes within the H-2I region and in man by genes within the HLA-D region. For cytotoxic T cells, B cells and perhaps suppressor T cells, antigen products of the mouse H-2K or D regions are involved and in man, products of HLA-A, B and C regions(106,108). The end result of this is that an animal may only be able to respond to extrinsic antigens for which it has Ia type antigens to associate. The finding that these HLA-D/DR molecules were not ubiquitous was even more intriguing.

D region antigens were found to be present on a limited number of tissues, initially macrophages, B lymphocytes, sperm and a few others were known(108). Further studies in kidney revealed the presence of HLA-DR antigens on several non lymphoid tissues. Endothelium of intertubular capillaries and glomeruli were found to contain substantial amounts and lesser amounts were present on larger vessel endothelium, glomerular mesangium and on "dentritic cells"(109). The importance of DR matching in renal transplantation thus became more understandable. Nevertheless Ia antigens are not ubiquitous. In skin the only cells that express these antigens are the Langerhans cells, a dentritic cell that functions as a fixed macrophage(110). A similar restriction in Ia bearing cell types was found in the pancreas and attempts to delete these cells began.

Minimizing Histoincompatability: Ex-Vivo Immunological Manipulation

Pancreatic endocrine cells have never been shown to express Ia antigens. Alejandro, *et al.* demonstrated that HLA-DR was located predominantly on vascular endothelium of both islets and exocrine tissues in adult human pancreas. Large amounts were also evident on macrophages and lymphocytes but due to the relatively small mass of these leucocytes, in pancreatic tissue, they considered endothelial cells at least as important a source of Ia antigens. Their monoclonal antibody technique showed an abscence of Ia on endocrine cells, exocrine cells and ductal tissues(111). Working with monoclonal antibodies to HLA-DR antigens in human fetal tissue, Danilovs *et al.* found HLA-DR cells to include a population of dendritic cells present throughout the exocrine regions as well as peripherally (through not centrally) in islets(112). Binding to large clusters of lymphoid cells in this fetal tissue illustrated graphically that fetal tissues can be expected to be at least as immunogenic as adult tissue. Other workers have demonstrated the presence of pancreas-specific alloantigens that seemed most likely to be enzymes or inhibitors secreted by acinar cells(113).

These studies demonstrated that Ia antigens were present on many cell types beyond passenger leucocytes. Further work demonstrated that these other cells could also effectively present antibodies to lymphocytes and so begin the effector phase of the immune response. Zitron _et al_. demonstrated the effectiveness of macrophages from peritoneal exudate in causing rejection of established islet allografts in rats(114). Hirshberg _et al_. showed that both epidermal Langerhans cells and umbilical vein endothelial cells can replace monocytes/macrophages as antibody presenting cells and were about equally effective(115).

The apparent lack of HLA-DR antigens from endocrine cells but its presence within islets means that even clean picked pancreatic islets devoid of exocrine contamination will incite an immune response and be rejected. Since even endocrine cells express the type I (HLA-A,B, & C) target antigens that are attacked by immune effector cells, sensitization of helper T cells by type II (HLA-DR) antigens on contaminant tissue will bring about graft destruction. But if current thoughts on immunological mechanisms are valid, a pure strain of pancreatic endocrine cells should be transplantable successfully with minimal or no immunosuppression(116). Many methods have been used in attempting to achieve this result.

Pretreatment of the donor with Ethionine(57) or alcohol can selectively destroy exocrine tissues and so reduce the immunological stimulus from the now more islet rich transplant tissue, but in vivo donor pretreatment will never be feasible in clinical transplantation, unless xenografting becomes a possibility.

A more practical approach to decrease the immunogenicity of islet transplants has been cell culture, with or without application of other modalities to eliminate passenger cells. Lacy et al. pretreated rats with radiation to eliminate leucocytes and silica to incapacitate macrophages, then cultured clean picked islets in 95% O_2 and 5% CO_2 at 37°C for 1 to 2 days, prolonging allograft survival across a minor histocompatability barrier(117). They could not duplicate these results across a major histocompatability barrier or when Ficoll separated cells (contaminated with macrophages, ductal, and vascular tissue) were used. When a single dose of antilymphocyte serum was added to the protocol for clean picked islets, survival across a major histocompatability barrier was achieved. Subsequent work demonstrated that 7 days of organ culture at 24°C and treatment with antilymphocyte serum achieved similar results; most probably by decreasing the number of, or altering, the passenger leucocytes(118). Lymphocytes from rejected islets showed a predominance of Ly 2+ cells (cytotoxic T lymphocytes are Ly 1-2+) while non rejected islets showed a predominance of Ly 1+ cells. These findings were believed by the investigators to reflect a decrease in cytotoxic cells and an increase in suppressor cells(119) around sucessfully engrafted islets.

Simeonovic et al. produced similar prolongation of islet allograft survival in mice with tissue culture for 17 days at 37°C, in 95% O_2 and 5% CO_2(120). They found the immunogenicity of fetal tissue more difficult to alter. Subsequent work showed the existence of a state of tolerance and resistance to rejection induced by specific stimulation of the recipient(121). However, Mandel et al. found that while human fetal islets grow well in conventional culture conditions and acinar cells die, under the conditions used to alter immunogenicity in mouse and rat islets (95% O_2 at 37°C), human islet cells were rapidly killed$^{(122)}$. Altering the culture conditions to those more suitable to human islets, Lacy et al. produced prolonged survival (>100 days in 70%, >200 days in 33%) of islet xenografts (rat to mouse) cultured at 24°C in air and 5% $CO_2$$^{(123)}$. Opelz and Terasaki had earlier demonstrated that low temperatue culture caused lymphocytes to lose the ability to stimulate an immune reaction$^{(124)}$. Rabinovitch et al. duplicated this prolongation of rat allograft survival by tissue culture and documented a 45% reduction in Ia content along with loss of identifable endothelial cells, macrophages and lymphocytes$^{(125)}$. Others have confirmed the loss of vascular endothelial cells as well as leucoyctes in cell culture$^{(126)}$.

Cell culture alone, under appropriate conditions, or more effectively when combined with antilymphocyte serum shows great promise in the alteration of immunogenicity in pancreatic islet transplants. The reduction of immunosuppressive medication doses or the complete elimination of a need for immunosuppression in human islet transplantation may indeed be possible. Unfortunately, the method by which cell culture techniques achieve these results are still not known; possibly because of this fundamental defect in understanding, cell culture techniques to alter immunogenicity have never been successfully applied to a large animal model. Other approaches are still required.

A fascinating alternative involves the use of Ia specific antisera and complement to treat islets. Studies with mouse islet allografts using these techniques have resulted in 100% survival of allografts for greater than 300 days. Faustman and co-workers believe the tissue bound dendritic cells that are rich in Ia antigen are the important cells deleted by this antibody treatment(127). Others have shown these Ia-positive dendritic cells to be ubiquitous among the Ia-negative parenchymal cells of liver, heart, kidney, thyroid, skin, and bladder(128), as well as pancreas, and it seems that these Ia-positive dendritic cells may be as much as 100 times more potent than lymphocytes at stimulating in vitro immune response. Faustman and group's achievement of allotransplantation without immunosuppression is truly a landmark study.

Work by Long and Scharp has taken another approach. Pancreas is being digested to a single cell suspension by sequential collagenase and trypsin digestion. The cells are then partially purified by centrifugation against a flow resistance, so called "centrifugal elutriation". Initial work has achieved a tissue product that is approximately 80% endocrine cells and autotransplantation by splenic reflux in dogs has resulted in normoglycemia in some animals studied(129). A solution that is 20% contaminants may not be satisfactory for allografting with minimal immunosuppression. Further work is ongoing involving flow electrophoresis separation of endocrine cells from contaminating acinar tissue, leucocytes, and endothelial cells to achieve a pure endocrine cell solution that may be transplantable without immunosuppression.

Immunoisolation Devices

Transplantation of allogenic and xenogeneic islets within semipermeable membranes has been attempted with the premise that a suitable diffusion barrier could let glucose and other nutrients into the islets and could allow efflux of insulin and other islet products while preventing cellular and perhaps even humoral immune responses from destroying the graft. Many variations of membranes have been tried. Early studies suffered from a massive fibrotic reaction from the host, effectively isolating the graft from satisfactory contact with body fluids(130). Recent work by Dr. A.M. Sun at the Connaught Research Institute has developed a membrane of polylysine sandwiched between layers of alginate. Studies with microencapsulated rat islets allotransplanted to the peritoneum have achieved normoglycemia for periods from 82 days to over 1 year(131). Large animal studies are lacking as of yet.

Implantation of the device within the bloodstream could improve the flux of nutrients and insulin but thrombosis and technical problems have prevented success for longer than a period of hours(132).

Blood Transfusions

Early in the history of renal transplantation blood tranfusions to a prospective recipient were avoided partly to prevent the sensitization of the patient to antigens that may have been present on a future donor kidney. Suprisingly, when studies were carried through, it seemed that kidney recipients who had previously received blood transfusions actually had better graft survival. It was initially though the beneficial effect was to be expected only from transfusions of blood from the donor, a possibility only with living related donors(133). Further studies revealed that the effect extended to recipients of cadaver kidneys that had received blood transfusions from unrelated people.(134).

A detailed analytic study by Terasaki, et al. documented that the main effect of transfusion was on reducing acute rejection episodes within the first months from the usual 30% to 10%(135). The effect was additive with HLA-A and B matching and maximal with HLA-DR matching at 2 loci. Blood transfusion is thus believed to produce enhancement or tolerance in recipients and so prevent the vigorous acute irreversible rejection process in about 20% of all recipients who would have experienced this reaction without transfusion, resulting in a 10% overall improvement in 1 year kidney transplant survival(136). Unfortunately, about 15% of transfused patients will develop high antibody titres, making future transplantation much more difficult. Recent advances in immunosuppression (Cyclosporine) have resulted in the loss of much of the benefit of the transfusion effect, at least in the short term, leading some experts to decry its further use because of the above mentioned problem of hypersensitization(137,138,139).

Recipient Immunosuppression

Tissue typing and organ banking has not reached the state where totally matched organs are available for every transplant recipient. Indeed, given the complexity of the major histocompatability complex, it may never be completely understood. New antigens are being found sporadically, such as the new non D/DR class II molecules encoded by the HLA-D region:MB/LB-E(140). Thus antigens distinct from Ia that are capable of stimulating allogeneic lymphocytes are a distinct possibility. Morrow and Sutherland have demonstrated rejection of mouse islets after transplant between animals identical at the H-2I region (class II - Ia - region alloantigens) but different at H-2K or H-2D(141).

Until the histocompatability of tissues is better understood or preparations of nonimmunogenic islet tissue are available for transplantation, immunosuppresion of the recipient will be necessary to prevent the rejection of transplanted allografts. This seems even more so in the area of pancreatic transplantation since the rejection of allotransplanted pancreatic or islet grafts has been more difficult to prevent than rejection of allografts of heart, skin, and parathyroid, as well as liver and kidney(142,143,144). Pancreatic islets have been demonstrated to produce a shorter period of normoglycemia after allotransplantation than whole organ or segmental pancreas transplants as well(145). It is not known if this is because collagenase digested islets are indeed more immunogenic (possibly enzymatic digestions uncovers otherwise "hidden" antigens) or whether the cellular clumps of transplanted islets are more susceptible to the effector mechanisms of rejection.

Recipient immunosuppression takes many forms. Non specific immunosuppression involves the unselective blocking of immune responses to foreign antigens without consideration of their origin: from allografted organs or from infecting micro-organisms.

The first step in the immune response that can currently be blocked is that of the macrophage actions of phagocytosing antigens, processing them and presenting them to lymphocytes for further action. Two drugs that alter macrophage function have been tested in pancreatic islet transplantation. Silica has been found to be very effective in preventing rat islet allograft rejection indefinitely if administration is begun 6 days prior to transplant(146). Carrageenan is also toxic to macrophages in vitro by the same mechanism as silica: the rupture of secondary lysosomes containing carrageenan (or silica) particles and enzymes toxic to the host macrophage (both are substances resistant to enzymatic destruction). Despite evidence of activity in several other allotransplant models, and despite the similarity of its method of action to silica, carrageenan (obtained from the marine alga chondrus crispus) has not been found effective in rat islet allotransplantation(147,148). Unfortunately, both compounds cause a permanent deficit in macrophage functions, an important example of which is thought to be tumor surveillance. The strong risk of greatly increased cancer rates has prevented any study of these agents in man.

Colchicine prevents replication of macrophages and polymorphonuclear leucocytes by binding to the tubular proteins of the mitotic spindle leading to cell death in metaphase. While it could conceivably have valuable usage in organ transplantation, colchicine also binds to the tubular proteins utilized by the pancreatic beta cell in the secretion of insulin, thus limiting its use in pancreatic islet cell transplantation(149).

Most of the immunosuppressive regimens currently used utilize lymphocytopenic agents. Various methods of destroying the cells involved in initiating (T helper cells), amplifying and effecting (cytoxic T cells, B cells) the immune response have been tried in pancreatic islet transplantation.

Ionizing radiation has been known to profoundly affect the immune system since the early 20th century. Unfortunately, whole body irradiation produced severe morbidity and frequently mortality when doses sufficient to produce immunosuppression were used. The subsequent use of total lymphoid irradiation therapeutically in Hodgkins disease, and the documentation of suppression of the immune system in patients treated so, rekindled interest in this modality. Investigation has revealed that the effect of irradiation is not uniform on all types of immune cells. It seems that cytotoxic T lymphoctyes are the most resistant to radiation, while T helper cells are very sensitive; the sensitivity of T suppressor cells is not firmly established but many believe them to be unfortunately more sensitive than helper cells(150). Total lymphoid irradiation combined with infusion of donor bone marrow cells to produce chimerism was used by Britt et al. to achieve tolerance to allogeneic pancreatic islets in rats without evidence of rejection or graft-versus-host disease for over 100 days(151). Najarian has recently published a protocol for total lymphoid irradiation combined with standard immunosuppression which resulted in fairly dramatic improvement in success with renal transplantation in patients who had previously rejected a kidney graft(152). As could be expected, however, morbidity with this method is great and mortality not insubstantial. Such a toxic method of immunosuppression must be reserved for the patient known to be at high risk for rejection and for whom no alternative therapy is available.

An agent without major toxicity in early tests is the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA). Adenosine deaminase inhibitors are more toxic to leuocytes than other cells. Because leucocytes lack the enzymes necessary for de novo purine synthesis, leucocytes rely on adenosine deaminase as a purine salvage pathway. By blocking this pathway, EHNA seems particulary effective against T lymphocytes. Lum et al have demonstrated prolongation of mouse islet allograft survival with this agent(153) and

Glazer was able to prolong mouse skin allograft survival with dCT, a related adenosine deaminase inhibitor(154), as were Ruers et al. with 2' deoxycoformycin(155).

Chemotherapeutic agents utilized in treating various malignancies have been found to have immunosuppressive effects. These include melphalan, 6-mercaptopurine, azathioprine, methotrexate, and cyclophosphamide. The main effect of the alkylating agent cyclophosphamide is exerted by its cytotoxic effect on lymphocytes, particularly B lymphocytes(156). The depression of humoral immunity which results has shown beneficial effects in renal transplantation, but little effect in pancreatic islets transplants(144,146). With the emergence of other less toxic, and more potent, immunosuppressives, cyclophosphamide is used infrequently at present in clinical transplantation.

Another chemotherapeutic agent, azathioprine, has been the backbone of clinical immunosuppression for transplantation for over two decades. In combination with prednisone, this was the first immunosuppression combination to give a reasonable expectation of survival for recipients of kidneys from cadaver donors(157). Results in canine and human whole organ and segmental pancreatic transplantation have not lived up to the results of renal transplants (52,158) and results in islet transplantation have been poor. Bell, et al. were unable to prolong normoglycemia in rat islet transplants with azathioprine either with or without steroids (146), nor could Kolb and associates in dogs (159). Kretschmer et al. using azathioprine and prednisone in canine pancreatic fragment allotransplantation achieved normoglycemia in only 8 of 24 dogs so treated.(160) All 8 subsequently died of infections felt to be due to excessive immunosuppression. Glucose tolerance testing in sucessful animals revealed significant abnormalities in the K value when compared to autotransplanted nonimmunosuppressed recipients.

The diabetogenic effect of steroids has always caused concern in pancreatic transplantation. With present yields islet transplants are not as efficient at maintaining carbohydrate metabolism as is a normally functioning pancreas; the added stress of steroids in transplantation could compromise graft function. Steroids possess a host of other undesirable side effects when used in high doses, the most serious of which has been an increased susceptibility to infection, especially when used in combination with other immunosuppressives. Infection has killed more patients following renal transplantation than has rejection, in large part due to the massive doses of steroids used to reverse acute rejection episodes. Nonetheless corticosteroids have been indispensible in renal transplantation for reversing acute rejection reactions and the Minnesota group has also found steroids useful for reversing rejection in segmental pancreatic grafts(161).

The prime mode of action of corticosteroids on the immune system is that of lysis of activated lymphocytes, although a myriad of other effects have also been noted(162). While it could be expected that steroids should provide a beneficial effect on islet transplantation as a single agent, Nelken _et_ _al._ could document no such effect in rats.(163) Bell _et_ _al_. could show no evidence of benefit of steroids when added to azathioprine in rat islet transplants (146) and others have substantiated the poor results of standard azathioprine/prednisone immunosuppression for both rat and dog islet models(159,160). Schulak tested intravenous methylprednisolone in rats for its ability to reverse acute rejection but could find no significant effect(164). Sutherland believed that high dose steroids might be detrimental to islet function(165). It may simply be that given the speed with which rejection can destroy transplanted islets, and the poor methods available to monitor rejection in islet transplantation (hyperglycemia is obviously a very late event), we may not diagnose rejection early enough for the lympholytic effect of steroids to be of any significant benefit.

One of the few agents that has shown promise of improving the results of azathioprine/prednisone treatment is antilymphocyte serum (ALS). This powerful immunosuppressive is produced from the serum of animals immunized with lymphocyte rich fractions of the sera of another animal, usually of a different species. The whole antiserum or conventional antilymphoctye globulin (ALG) derived from the serum is very effective in depleting circulating lymphocytes, presumably by coating and opsonizing them for macrophage ingestion, or by causing complement-mediated lysis(166). T-lymphocytes seem to be affected more than other cell lines. ALS has been shown to prolong survival in animal allografts of kidney, liver and heart(167). Evidence has been conflicting, however, on the results of ALS on human transplants of these organs(166), partly due to variation in the activity of ALS from various sources, and partly due to variation in dosages utilized. Double blinded, randomized trials have shown either clear benefits from ALG(168) or no benefit(169).

In islet allografts, ALS has shown strong evidence of immunosuppressive activity in animals. Frangipane showed prolongation of mouse islets allograft survival(170) and Beyer and Friedman prolonged rat islet allograft survival(171). Barker _et al_. demonstrated prolonged survival of rat to mouse islet xenografts with ALS alone(172). Combinations of ALS with cell culture have resulted in prolonged islet allograft survival despite no further ongoing immunosuppression(118). Lorenz _et al_. achieved prolonged normoglycemia in dogs with a weak histoincompatability by prolonged immunosuppresion with azathioprine and anti-thymocyte serum(173).

Antilymphocyte serum is not without problems however. Allergic reactions have been troublesome(167) and excessive immunosuppression in protocols using ALS in combination with other immunosupressive drugs has been blamed for a high incidence of lymphomas (mainly of EB virus type) in humans following heart transplants (especially for cardiomyopathy) and renal transplants. Thrombocytopenia and leucopenia are common side effects of ALG, requiring discontinuation of therapy in some patients(169).

A very interesting and related approach has been the use of monoclonal antibodies against T cells. The technique of production of monoclonal antibodies from hybridomas was developed by Kohler and Milstein(172) and the OKT3 monoclonal antibody has been used clinically, though not yet with dramatic effects, partly because of the tendency of the recipient to develop anti-mouse antibodies(173). Cosimi was able to reverse initial acute rejection episodes in 8 patients with renal allografts but subsequent further acute rejection episodes occurred in 5 of the patients and were irreversible in 2(174). More recent studies have shown powerful effects in reversing steroid resistant rejection episodes, but again subsequent rejection episodes were not uncommon, and some were irreversible. Severe diarrhea with the initial IV dose is very common and wheezing, fever and chills not rare(175). Production of monoclonal antibodies using human-cell hybridomas could be a major advance in coming years.

Semi-Specific Immunosuppression:

An agent with a substantially different mode of immunosupression, which I will call "semi-specific", is cyclosporin A (CsA). CsA was discovered in the Sandoz Research Laboratories in Basel, Switzerland by Dreyfuss *et al*. during screening of fungal metabolities for antibiotic properties. It was a poor antibiotic but the demonstration of delay in rejection of H-2 incompatible skin grafts in mice by Borel led to further investigations(149).

Cyclosporin A is a cyclic endecapeptide of molecular weight 1202, composed of 11 amino acids, one of which was previously unknown. It is one of a family of similar peptides produced by soil fungi, in this case Tolypocladium inflatum Gams. It is intensely hydrophobic but is soluble in ethanol, olive oil, medium chain triglyceride or tween 80 surfactant (Sigma Chemicals, St. Louis, Missouri). Due to its hydrophobic nature, absorption after both oral and IM administration has been variable. A formulation for intravenous use is now available and the use of olive oil as a vehicle has improved oral absorption somewhat. With oral administration, maximal plasma levels are reached within 3 to 4 hours and T $\frac{1}{2}$ is from 17 to 40 hours(176). The initial therapeutic dose in man is 10-20 mg per kg body weight daily. Toxic effects are minimized by maintaining trough levels in plasma below 400 mg/l(177). Most of the drug is metabolized by the liver and excreted in bile. About 10% is excreted by the kidneys, mainly in the form of metabolites, only 0.1% is excreted unchanged by the kidneys(176). Obviously alterations in liver function could dramatically affect serum concentrations, and thereby alter immunosupressive actions and toxicity(173).

Determination of serum CsA can be performed rapidly by high performance liquid chromatography or by a radio immunoassay of inhibition caused by test serum between a reference serum and ^{3}H-labeled CsA. The reliability of these tests is not yet absolute and the relationship between serum level, as measured by either of these methods, and the immunosuppressive action of the drug has not been established(178). Toxic levels are better known, at least in the human situation.

A major advantage of CsA is its apparent lack of myelotoxicity(179). Most other drugs with significant immunosuppressive actions have been toxic to bone marrow(176) including the present standards azathioprine and Antilymphocyte globulin(180). Early studies demonstrated the ability of CsA to selectively kill those lymphocytes that can destroy allografts without totally destroying the immune system(180). Further investigations suggested that CsA was non toxic to small lymphocytes but highly toxic to lymphoblasts generated by mitogens or allogeneic cells(181). Calne proposed a selective elimination of helper T cells(182).

Gordon and Singer, publishing in Nature(180) pursued the mode of action of CsA whereby it avoided myelotoxicity. Using colony formation as an endpoint, he demonstrated far greater toxicity toward T cells than toward B cells or hematopoeitic stem cells. His results also suggested subpopulations of T cells, some sensitive to CsA and some not.

Wang, utilizing both in vitro and in vivo methods, showed that CsA, added early on to mixed lymphocyte culture, rendered lymphocytes incapable of being activated by re-exposure to alloantigens present in the MLC initally, while the ability to respond to unrelated antigens was seemingly intact. He could show no effect on cytotoxic lymphocytes already generated and so suggested that CsA acted on the induction of T killer cells rather than on the effector phase of the immune response itself(183).

Foa et al, using mixed lymphocyte culture techniques had similar results. Overnight incubation in as little as 1 μg/ml of CsA resulted in 60% inhibition of proliferating (bone marrow) T cells while concentration of CsA of 10 μg/ml could not inhibit colony formation by resting (peripheral blood) T cells(184). Kunkl confirmed that while virgin T helper cells were sensitive to CsA, lymphocytes already primed by alloantigens were not. Since CsA had little effect on unstimulated cells it would have little effect on ongoing antibody production. Kunkl also demonstrated a selective action of CsA on some B cells. A certain population of B cells that were responsive to T-independent antigens were exquisitely sensitive to CsA, while other B cells responsive to T-independent antigens and all T-dependent B cells were resistant to the drug(185).

Recent work has done much to explain the actions of CsA that can produce this selective action. It is clear that CsA exerts its effect early in the immune response, it is not effective in preventing rejection if given to the recipient after T cells have been primed by foreign antigen (186)). This work by Bunjes et al. and an elegant series of experiments by Palacios _et al_.$^{(187)}$ has thrown much light on the cellular and the subcellular actions of CsA. A brief review of current thoughts concerning the method by which alloantigens stimulate a rejection reaction is helpful.

T lymphocytes activated by either soluble antigens, particulate alloantigens or mitogens recognize at least two distinct sets of factors for clonal expansion and maturation. Alloantigen is presented in association with HLA-DR antigens by accessory cells (macrophages) to T cells. This primary event results in several changes. First, receptors for Interleukin-2 (Il-2), previously named T cell growth factor, are expressed by Il-2 reactive T cells. Second, antigen triggered cells signal adjacent accessory cells to start producing Interleukin-1 (Il-1), previously known as lymphocyte activating factor, and also begin expressing receptors for Il-1 themselves. Only in the presence of Il-1 will the activated T cell begin production of Il-2. T cells apparently depend absolutely on Il-2 for continuous growth, but both the production of Il-2 and expression of sensivity to it require a primary activation of the T cell by antigen or mitogen$^{(176)}$.

Cyclosporin A stops the proliferation of T cells induced by either antigens or mitogens by actions on 3 distinct actions on this cascade:

(a) inhibiting T cell help to accessory cells for the synthesis of interleukin - 1.

(b) preventing the Il-2 producing T cell from expressing receptors for Il-1 and so suppressing synthesis of Il-2.

(c) rendering T cells unresponsive to Il-2(176,186,187).

Several of these aspects of the mode of action of CsA promise a new level of immunosuppression for organ allotransplantation. The apparent lack of myelotoxicity is a dramatic improvement over previous immunosuppressive agents. The drug has not proved to be carcinogenic *in vitro*, unlike both azathioprine and cyclophosphamide. The limited site of action on the immune system is another major advantage over previous agents whose action was achieved by non selective destruction of lymphoid cells, with or without other tissues. CsA has been demonstrated to largely spare B lymphoctes from limitation to function; committed T lymphocytes producing antibody are likewise spared. No adverse effects have been found on macrophage phagocytosis and effects on natural killer (NK) cells were not marked. NK cells (thought to be important in immune surveillance against tumors) are not directly affected by CsA, but CsA does decrease T cell production of gamma interferon, a positive modulator of NK cells(176). Lower risks of overwhelming infection should thus be attainable with CsA as compared to previous immunosuppressive agents.

The cellular and sub-cellular mechanisims of action are slowly being ellucidated for Cyclosporin A. This should allow more logical therapy and a better understanding of failures. An example being that CsA therapy should be started prior to transplantation and, in most species, therapy must be continuous to achieve long graft survival. Perhaps even more importantly, a knowledge of mechanisms of action may allow development of related compounds with even more potent and more selective action on the immune system, and accompanied by less toxicity.

A review of the results achieved to-date with CsA immunosuppression in organ transplanation is now in order.

Application of CsA immunosuppression to organ transplantation in animal models has been impressive. The first results were by Kostakis who demonstrated that short-term CsA therapy resulted in prolonged or indefinite survival of allogeneic rat heart allografts(188). Subsequent work showed indefinite survival of rat renal allografts after a short course of CsA(189). Other rat studies showed potent immunosuppression in liver, skin and bone narrow transplantation(182). Similar prolonged survival of grafts after cessation of CsA in rabbit kidney(190) and pig orthotopic heart allografts(191) led to speculation that CsA had its effect by specifically deleting the clone of lymphocytes reactive to the graft. Further studies in other species and with other organs, as well as studies of the cellular and sub-cellular mechanisms of action of CsA have refuted these claims. A more logical answer seems to be the development of a state of tolerance due to suppressor cell activities in these long surviving grafts.

Further work with renal transplantation in rhesus monkeys(192) and dogs(193) substantiated the potent immunosuppressive effects of Cyclosporin A, but organs were rejected shortly after therapy ceased. Human studies(193) also demonstrated a cumulative effect on immunosuppression of Cyclosporin A in combination with prednisolone and azthioprine, with suggestion that this latter combination could be the most potent.

Cyclosporin A was found by Norin et al. to be effective as immunosuppression in canine lung allografts(194). They believed additional steroids would still be necessary to prevent chronic rejection and found that acute rejection episodes were easier to suppress in the CsA treated dogs than in conventionally immunosuppressed animals. They likewise found a CsA/azathioprine combination to be the most effective immunosuppression. Veith et al. were also successful with CsA in lung allografts in dogs(195) and Reitz et al. used CsA with success in monkey heart-lung transplantation(196).

The results of CsA therapy in human organ transplantation have also been impressive. Several large randomized prospective trials have demonstrated a 20% reduction in rejection in the first year following transplantation when CsA with or without prednisone is compared to standard azathioprine-prednisone immunosuppression. One year graft survival in the European Multicentre Study improved from 52% to 72% in the group of 232 patients randomized(197). The Canadian multicentre trial of 210 patients showed an 80.4% to 64.0% advantage for CsA(198) and Starzl's trial in 70 patients found a 90% to 50% advantage for CsA(199). As in many studies, Kahan in Houston documented fewer episodes of acute rejection when renal transplant patients were treated with CsA; he also demonstrated a decrease in helper T cells relative to suppressor T cells in patients found preoperatively to be strong responders(200).

Not all studies of CsA in renal transplant patients have illustrated an improvement in graft survival. The most recent randomized trial of 182 patients from Minnesota showed no improvement(201), nor did Sheil's group in Australia with 60 patients randomized(202), nor the Tuebingen, Germany study of 65 randomized renal transplants(203). Halloran extracted results from 4 Ontario transplant centres from those of the entire Canadian study and saw no improvement with CsA(204). Halloran also pointed out that all of these studies that did not achieve an improvement in 1 year renal transplant graft function with CsA and prednisone were centres that already had graft survivals of 70 to 80% at 1 year. All of these centres were employing some form of prophylactic antilymphocyte globulin (ALG) with their standard azathioprine-prednisone treatment to achieve these results, and it is perhaps not surprising that these relatively small studies could not demonstrate a statistical improvement in graft survival over these already excellent 1 year figures.

Oyer compared 99 consecutive heart transplants performed by Shumway's Stanford group from 1976 to 1980 that were immunosuppressed with azathioprine-prednisone to their first 66 patients treated with CsA and found 1 year patient survival improved from 63% to 82% and 2 year survival from 56% to 74%(205). Rejection episodes were not significantly decreased in number but the severity of the episodes was: only 1 patient died from rejection in the CsA group while 10 did so with azathioprine. Episodes of infection likewise were much less severe with CsA and mortality from infection decreased from 40 of 99 to 5 of 66 patients. Ninety percent of the CsA treated patients were able to resume a normal life, only 70% of the former azathioprine group have done so. The Stanford group's results have undergone such a dramatic improvement since the availability of CsA that a major resurgence of interest in cardiac transplantation has occurred with all centres now utilizing CsA and prednisone.

Combined heart-lung transplatation has likewise achieved some excellent results with CsA. Jameson of the Standford group reports a

74% 1 year and 58% 2 year survival in patients transplanted for Eisenmenger's syndrome(206).

Williams et al. were impressed with the results of CsA in canine liver transplants. Survival was more than double that of azathioprine treated animals, and no CsA treated dog rejected his graft (histologically or biochemically) until the dose of CsA was reduced to <20 mg/kg daily(207).

One hundred and seventy patients transplanted by Starzl at Denver and Pittsburgh over a 17 year period achieved 1 year of survival in only 33% of cases, with no significant improvement in later years(208). In the 224 consecutive patients transplanted with CsA and prednisone since that time, 1 year survival has been 68%. This improvement has been evident in nearly all subgroups of patients from biliary artresia in children to sclerosing cholangitis(209). In the words of Starzl liver transplantation has now evolved from a "highly unpredictable experimental undertaking to a bona fide service" since the introduction of CsA.

Cyclosporin has also been applied to transplant organs which had not successfully been transplanted in animals immunosuppressed with other agents. Craddock et al. achieved a mean survival of >100 days in dogs with total small intestinal allotransplantation when CsA was administered intramuscularly for 30 days, then orally(210). Ricour et al. achieved survival of small bowel allografts in piglets beyond 200 days with CsA administered intravenously for 5 to 10 days, then orally. A CsA level of > 250 mg/ml in serum was found necessary to prevent rejection(211). CsA has been capable of preventing graft versus host disease in small bowel transplantation as well(212,213). Black reported survival of whole rat limb allografts for a median 101 days(214) and Furnas achieved indefinite rat whole limb survival (> 400 days) with CsA(215).

Recent human studies have brought to light many side effects of CsA that were unrecognized in early animal toxicity studies. Minor problems have been many, including: hirsuitism, gum hypertrophy, tremor, paresthesias, benign breast tumors, hyperkalemia and intestinal ileus.

Conventional immunosuppression in transplant patients results in a level of malignancy about 6 times that of the normal population. Experience with CsA now suggests a similar incidence of malignancy but over 80% of CsA associated malignancies have been lymphomas(216). Early work by Calne at Cambridge found a 10% incidence of lymphoma often in the gastrointestinal tract(217). The Stanford group reported 4 lymphoid malignancies in their first 28 cardiac transplant patients treated with CsA, all of whom had also received rat antithymocyte globulin(205).

The majority of these tumors can now be shown to be associated with Epstein-Barr virus(EBV)(176,218). Many are not true malignancies but rather abnormal proliferations of B lymphocytes due to transformation by EBV infection in the face of high levels of immunosuppression(176). Nanto has proposed a useful classification of lymphoid proliferations in immunosuppressed patients based on the gross appearance of the involvement, the morphological appearance and whether the lymphocytes are polyclonal or monoclonal. Treatment of benign appearing polyclonal tumours with acyclovir has been effective by preventing EBV proliferation and infection of other B lymphocytes and so stopping transformation. Polyclonal tumours with abnormal morphology require a decrease in immunosuppression to avoid further progression to frank monoclonal lymphoid malignancies which present as solid tumors and have a poor response even to aggressive treatment with surgery, irradiation and chemotherapy(219). Recent clinical work has documented the usefulness of this approach and the greatly improved prognosis of these polyclonal "pseudolymphomas" that represent the majority of lymphoid proliferations in CsA treated patients(220). With lower levels of immunosuppressive therapy, monitoring of serum CsA levels to minimize dosages, and improved understanding of the nature of these diseases, current studies show no increase in malignancy when CsA is used when compared to standard immunosuppression(221,222,220).

The most serious complications of CsA therapy encountered thus far has been nephrotoxicity. CsA is believed to be physiologically toxic to the tubules, consistent and specific microscopic changes have not been documented(223). This causes much concern in renal transplantation where an increasing creatinine could be due either to excessive CsA or insufficient immunosuppression and subsequent rejection. Fortunately, the nephrotoxicity is dose related and responds to either a decrease in dosage of CsA or its stoppage and substitution by standard immunosuppression. The renal effects have been reversible even after prolonged (>6 months) evidence of nephrotoxicity in both animal and human models(224,173), and no progressive structural changes have been documented(173). A controlled trial of patients experiencing cyclosporin nephrotoxicity at the University of Minnesota found the approach most likely to maintain graft function (88% to 2 years) was to lower the CsA dose, and if creatinine did not improve, to drop CsA dose further to 5 mg/kg/day while adding a low dose of azathioprine to prevent rejection(225).

Mild hepatotoxicity has been seen with both renal(221) and hepatic allotransplants(122). All episodes of hepatotoxicity seen in the Canadian Multicentre Trial occurred with serum trough CsA levels > 700 mg/L(226). Changes in recent studies have been limited to mild to moderate increases in bilirubin and alkaline phosphatase with no serious problems. Nevertheless, any degree of hepatotoxicity due to CsA or other factors could cause serious build up in serum levels in this drug which is totally dependent on hepatic metabolism for inactivation. Liver function tests should be followed regularly along with renal parameters in all patients on CsA therapy.

Another problem which surfaced in early studies was that of infection. Brent documented several severe infections, the most serious due to herpes virus pneumonitis(227). Others have also documented viral infections(207) and the mechanism is likely similar to the inhibition of T cell control of viral replication in B cells that is thought to be responsible for EB virus associated tumors(176). The Oxford group found no increase in infection when CsA was added to steroid immunosuppression(193). A review of the extensive experience with the drug at Cambridge revealed no major problems with bacterial infections and the only frequent viral problem was a reactivation of certain latent viruses, most notably Epstein-Barr and other Herpes type viruses(191). Certainly the mechanism of action of Cyclosporin A would lead one to expect a lower incidence of infections that would use of standard immunosuppression. More recent work with lower but still effective doses of the agent seem to have born out this expectation(228,189).

Several workers have demonstrated that CsA can be safely changed to standard immunosuppression in human renal transplantation or vice versa(189,173). The immunosuppressive effect of CsA was found to be synergistic with several other medications, notably azathioprine and prednisone(229). Toxicity in combination with cyclophosphamide or triple therapy with azathioprine, prednisone and CsA has been found to be excessive by some centres (230). Ringden et al. found a high incidence of serious infections in human renal transplant recipients(231) but others could not document cumulative toxicity with CyA and either prednisone or azathioprine in canine renal transplants(193). Although some degree of cumulative toxicity would be expected to accompany the synergistic effect when CsA is used with other immunosuppressive agents, the Minnesota group has demonstrated that CsA can be safely combined with prednisone or azathioprine and prednisone for long periods(161,228,225,52).

The results of cyclosporin in pancreatic transplantation have been less dramatic, partly because of the inherent difficulties with the technical aspects of whole organ transplantation, and, also because of problems in achieving sufficient yields and adequate engraftment in islet models.

Garvey et al. achieved prolonged survival of segmental allografts in rats using a high dosage of CsA but were unable to do better than a slight improvement in survival of islets infused into the liver. Similar results were obtained with allogeneic fetal pancreas implanted under the renal capsule(232). Rynasiewicz et al. achieved similar results in rat segmental allografts and while they achieved some success with rat islet transplantation across a minor histocompatiability barrier, even with huge doses they could not prevent rejection of islets transplanted across a major barrier(233). Subsequent investigation by these workers failed to achieve significant results in rat pancreatic islet transplants (even with combinations of CsA with prednisone or azathioprine) when a major histocompatability difference was involved, unless toxic doses that resulted in eventual death of the animals were used(228,229). Vialettes *et al.* on the other hand, achieved significant prolongation of rat islets allotransplanted across a major histocompatability barrier and illustrated the occurrence of successive and incomplete rejection episodes before definite rejection, a valuable finding with reference to clinical transplantation(234).

Canine pancreatic transplantation has achieved similar mixed results. McMaster, Procyshyn, et. al. did not prolong normoglycemia or survival of dogs with segmental pancreatic transplants using azathioprine and prednisone immunosuppression. CsA in doses of 18 and 25 mg/kg daily was able to achieve normoglycemia for means of 18 and 55 days respectively, but not indefinitely(158). DuToit *et al.* at Oxford achieved indefinite (>100 days) graft survial in 5 of 8 dogs with segmental allografts given a dose of 40 mg/day but only slight prolongation of graft survival with 25 mg/kg/day(235).

Results with human pancreatic transplantation remain inferior to those achieved with CsA in renal, hepatic, and even cardiac allotransplantation. Technical problems, especially vascular thrombosis and exocrine enzyme leakage, still results in a large number of failures and rejection destroys most of the technically successful grafts within the first year. Of technically successful grafts reported to the Clinical Pancreas and Islet Transplant Registry only 26% of azathioprine-prednisone treated grafts have functioned for 1 year.

Cyclosporin A has improved that figure only to 41% 1 year graft function when technical failures are excluded(56), rejection still causing most of these losses(46).

Human islet transplants have been even less successful: of 116 attempts since 1970, none were functioning as of June 1982(56). Only 4 were ever claimed to have had prolonged function, and only one of these was well documented. The patient of Largiader and Kolb in Zurich who underwent an islet transplant and kidney transplant from a 4 year old cadaver donor and was maintained without insulin for 19 months despite recurrent acute rejection episodes. She was immunosuppressed with antilymphocyte serum, a short course of cyclophosphamide and maintenance azathioprine and prednisone, but ultimately rejected her kidney and became hyperglycemic and died 1 month later(74).

These poor results must be tempered somewhat: methods for isolating pancreatic islets have improved considerably from the small yields of large particles available during these trials(79,80) and indeed many of these islet transplants were not even immunosuppressed(73). Four cases of allotransplantation of cultured fetal pancreata immunosuppressed with CsA have been reported from 2 Australian centres, but no long term function was reported(56). No attempts at pancreatic islet transplantation in humans utilizing Cyclosporin A immunosuppression have been reported(56).

Results in canine islet allotransplantation have been similarly bleak thus far. Sutherland et al. were able to achieve only minor prolongation of survival in 8 of 24 islet allografts with standard azathioprine/prednisone immunosuppression(165), Kolb found no significant effect(159). DuToit was unable to achieve normoglycemia postoperatively in either nonimmunosuppressed allografted dogs or allografted dogs immunosuppressed with CsA (25 mg/kg/day), despite achieving prolonged normoglycemia in autografted animals(236). Mahoney, Merrell, et al. was unable to prolong pancreatic islet allograft survival in dogs despite using doses up to 40 mg/kg/day(237). Williams achieved normoglycemia for over 60 days in 2 of 4 dogs treated with oral CsA at a dose of 40 mg/kg/day. The dogs were littermates that were compatible for transplant by serological (dog lymphocyte antigen-DLA-typing) and cell mediated assays (mixed lymphocyte culture-MLC). When CsA was administered orally at 25 mg/kg/day or when non littermate dogs were allografted, euglycemia was not achieved(238).

Sutherland has stated that a satisfactory test of islet allografting must achieve normoglycemia for 2 days before comment on rejection can be made. This will ensure that satisfactory engraftment and function of the islets has been achieved in the host to allow a fair evaluation of immunosuppression to be carried out. No study of canine islet allotransplantation testing Cyclosporin A immunosuppression has consistently achieved this goal. Several changes in technique and approach may help to do so:

1. The technique of Horaguchi and Merrell, especially as modified by Warnock et al. has been shown to be superior to earlier methods of islet isolation(79,80).

2. Transplantation by splenic venous reflux has been demonstrated to be more efficient than direct splenic puncture(80).

3. Cyclosporin A as an oral agent is absorbed less efficiently by some patients and absorption of orally administered CyA could be expected to be poor in the perioperative period when total pancreatectomy has been carried out. Intravenous administration in the perioperative period should overcome any difficulty.

4. Monitoring of Cyclosporin A plasma levels should demonstrate insufficient levels that could results in rejection or high levels that could cause toxicity.

The major thrust of study of this project will be to optimize conditions for canine pancreatic fragment allografting to allow a fair evaluation of the ability of Cyclosporin A at nontoxic doses to prevent rejection of transplanted islets.

IV MATERIALS & METHODS

Animals and Care

53 conditioned outbred, unrelated young adult dogs (<2 yrs.) weighing 15-25 kg and of varying breeds and crosses were studied. The animals were housed in the large animal Vivarium of the Surgical-Medical Research Institute at the University of Alberta. They were under the care of the investigators with the assistance of the animal care staff and the advice of a veterinarian, according to the criteria of the Canadian Council on Animal Care.

The animals were allowed unrestricted exercise twice daily and were fed a full diet of 360 g of meat (Dr. Ballard's) and 600 g of Pow R Pac Burger Bits. The dogs were fasted overnight, then under general endotracheal anesthesia underwent a total pancreatectomy. Intraoperatively approx. 1000 ml of Ringer's lactage was given intravenously and hydration was maintained postoperatively with 1000 ml of Ringer's lactate subcutaneously on the first post op day and 500 ml on the second. The animals were NPO on Day 1 post op, were allowed water ad lib on Day 2, meat supplemented with 12 capsules of cotazym (Organon) on Day 3 and a full diet supplemented with cotazym thereafter.

Dogs 15-20 kg received 3 ml of Pendistrep (Rogar/STB; 600,000 IU procaine pencillin G and 750 mg dihydrostreptomycin sulfate) IM preoperatively and 3 ml Derapen C (Ayerst; 300,000 IU benzathine Pen G, 300,000 IU procaine Pen G, 750 mg dihydrostreptomycin sulfate) IM on Day 1 post op. Dogs 20-25 kg received 4 ml of each solution on the same schedule.

Where storage with cryopreservation delayed reinfusion of islets and resulted in a second laparotomy, 10 u of NPH insulin daily was given subcutaneously exclusive of operating days and the animals were allowed water on the first day after the transplantation with meat and full diet (each with Cotazym) on days 2 and 3 postop respectively.

Any dog that developed severe metabolic complications or other incurable problems was sacrificed with an overdose of anesthetic. These and all animals that died were autopsied to ascertain cause of death, to insure completeness of pancreatectomy and to obtain tissue for histology.

Pancreatectomy

After an overnight fast the animals were given 32 mg/kg sodium pentobarbital, endotracheally intubated, an intravenous line established, and the abdomen prepped with a povidone-iodine solution. A midline abdominal incision was made and the pancreas was mobilised with preservation of its vascular supply. The L and R pancreatic ducts were cannulated (PE90 and PE50 polyethylene tubing respectively), a sample taken for insulin and amylase assay and the gland removed with care to avoid leaving any pancreatic remmants insitu. The gland was immediately weighed, then distended with 80 ml chilled Hanks balanced salt solution (HBSS) immersed in cold HBSS and transported on ice for immediate processing.

For apancreatic control animals and those involving cryopreservation and delayed transplant the abdomen was closed and the animal returned to the Vivarium. For acute autotransplants and allotransplants the animals were maintained under anesthesia by an assistant until the processed pancreatic fragments were available for transplantation.

Pancreatic Dispersed Fragment Preparation

1. Digestion. Preparation was accomplished by a modification of the method of Horaguchi and Merrell that had been developed by Warnock *et al.* at this Institute(80) (Fig. 1). The ductal system of the gland was perfused with cold HBSS (10°C) for 10 min. The perfusate was then changed to a 0.4% solution of collagenase (Sigma Chemicals, St. Louis, Missouri; Type I lot #121F-0517) prepared by adding 400 mg of collagenase powder to 100 ml HBSS, then sterilized by passing through a 0.22 μ filter. Collagenase digestion proceeded at approximately 30°C with a flow rate of 20 ml/min and a perfusion pressure that began at 250-400 mmHg and fell progressively with digestion to 150-250 mmHg. Adequate digestion was gauged by visual inspection of the gland, achievement of a soft, mucoid consistency to the gland and easy fragmentation of the acini with forceps.
2. Mincing. The digested gland was then divided in two and immersed in an enzyme stop solution of HBSS with 200 KIU Aprotinin (Trasylol, Miles Pharmaceuticals, Rexdale, Ont.) 100 units K^+ Pen G and 100 μg Streptomycin sulfate per ml. This solution was used in all subsequent steps (unless otherwise indicated). The gland was then minced with mechanical rotating blade for 90 sec., during which time large duct fragments were manually removed.
3. Dissociation. The resulting suspension of pancreatic fragments was further dissociated by vigorous shaking for 10 minutes in an Evapo Mix shaking water bath at 0°C. The tissue was then filtered through a 400 μ screen with unfiltered tissue being returned to the shaker twice before discarding the remaining

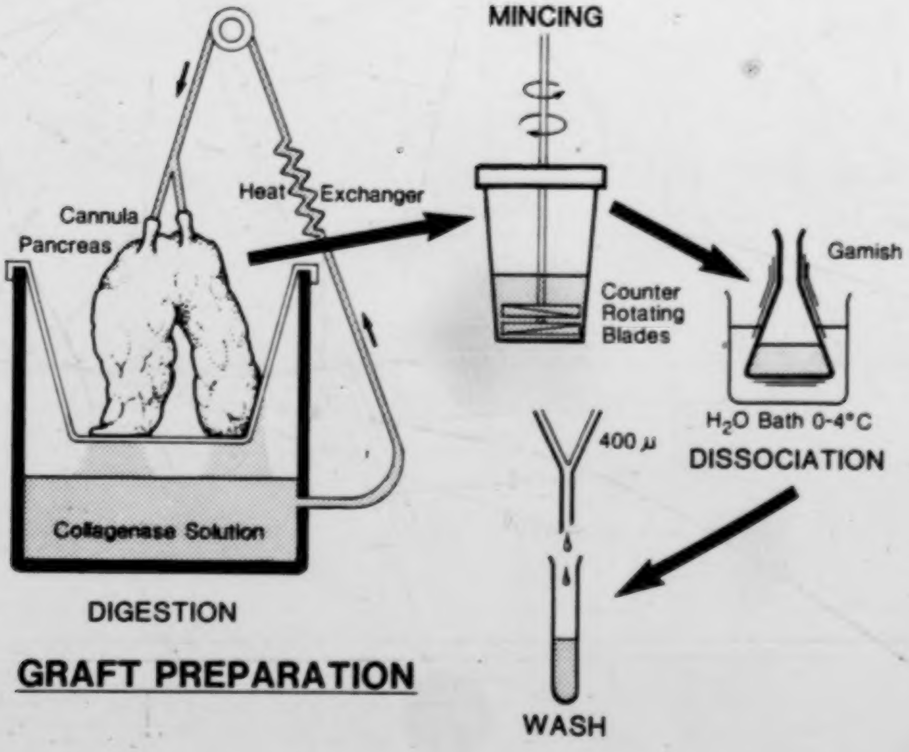
MINCING
Cannula
Pancreas
Heat Exchanger
Counter Rotating Blades
Gamish
H_2O Bath 0-4°C
DISSOCIATION
400 μ
Collagenase Solution
DIGESTION
GRAFT PREPARATION
WASH

FIGURE 1. PANCREATIC DISPERSED FRAGMENT PREPARATION.

ducts and large acinar tissue clumps.

4. Washing. The suspension, now containing pancreatic fragment <400 μ diameter was washed three times by centrifugation at 800 G, 450 G, and 450 G, aspiration of the supernatant and resuspension of the pellet. Samples of the final post-isolation pellet were taken for insulin and amylase assay and histology. The pellet was then resuspended with cold HBSS to total 30 ml and placed on ice for acute transplantation or resuspended in a tissue culture media at room temperature for cryopreservation.

Processing lasted approximately 2.5 hours. Most steps were carried out in a laminar flow hood and attention to aseptic technique was maintained throughout processing.

Cryopreservation (Fig. 2)

Following isolation the final pellet was resuspended in an equal volume of room temperature (22°C) Medium 199 (Gibco, Grand Island, N.Y) with 10% fetal calf serum (fcs), 2% Aprotinin, and 0.4% PenG and Streptomycin (as added to HBSS). A third equal vol. of 2 M dimethyl sulfoxide (Me_2SO) in the same Medium 199 mixture referred to above was then added to the Erlenmeyer flask which was gently swirled in a water bath at 25°C for 5 min. Another vol. of 2M (Me_2SO) solution was then added and shaking continued for 25 min. Four volumes of 3M Me_2SO soln. were added (final Me_2SO concn. in islet bearing solns was 2M) followed by shaking at 25°C for 15 min., then cooling to 0°C while shaking for a further 5 min (the first 8 were not cooled to 0°C).

The tissue suspension (0.5 ml tissue in 4 ml suspension) was then aliquoted to 16 x 125 mm glass KIMAX test tubes while kept on ice. The

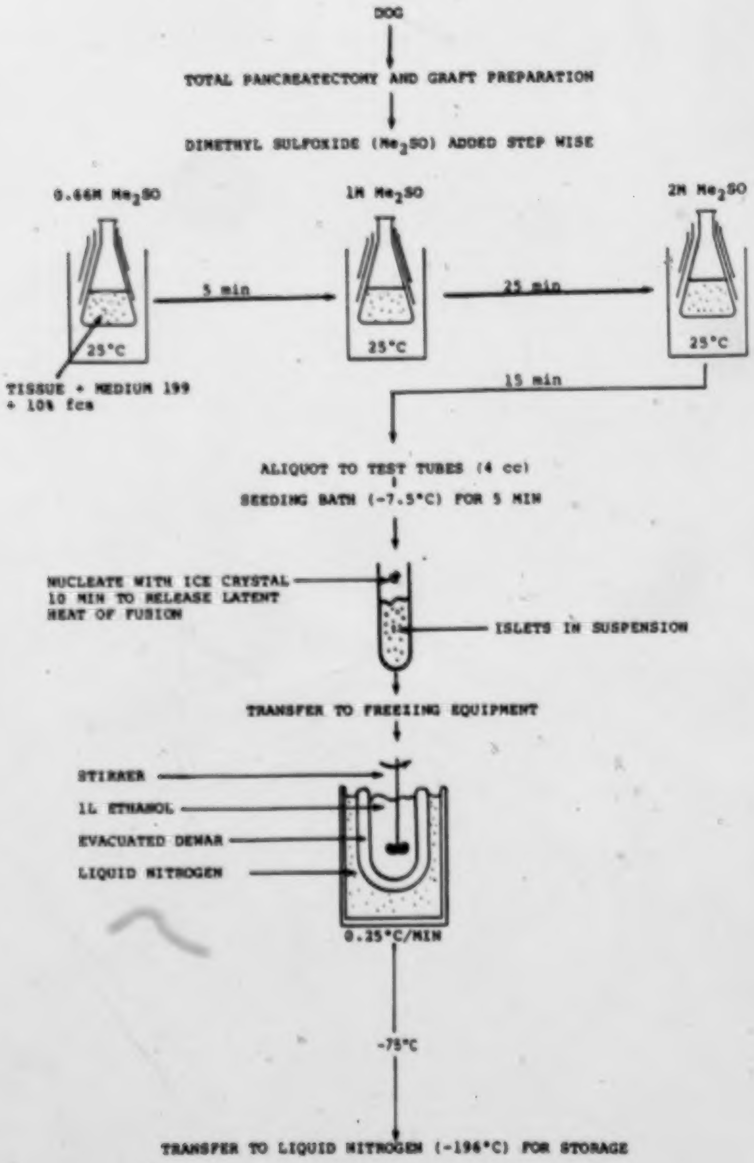
DOG
TOTAL PANCREATECTOMY AND GRAFT PREPARATION
DIMETHYL SULFOXIDE (Me$_2$SO) ADDED STEP WISE
0.66M Me$_2$SO
1M Me$_2$SO
2M Me$_2$SO
5 min
25 min
25°C
25°C
25°C
TISSUE + MEDIUM 199
+ 10% fcs
15 min
ALIQUOT TO TEST TUBES (4 cc)
SEEDING BATH (-7.5°C) FOR 5 MIN
NUCLEATE WITH ICE CRYSTAL
10 MIN TO RELEASE LATENT
HEAT OF FUSION
ISLETS IN SUSPENSION
TRANSFER TO FREEZING EQUIPMENT
STIRRER
1L ETHANOL
EVACUATED DEWAR
LIQUID NITROGEN
0.25°C/MIN
-75°C
TRANSFER TO LIQUID NITROGEN (-196°C) FOR STORAGE

FIGURE 2. CRYOPRESERVATION PROCEDURE.

tubes were capped in the laminar flow-hood, then transferred to an Ethanol bath at -7.2°C to supercool for 5 min. To prevent wide and rapid temperature swings with freezing, the tissue was then nucleated by contacting the tube with a cold brass rod (first 8) or copper tubing through which liquid nitrogen (N_2) was pumped by an FTS circulating pump. Fifteen minutes were allowed for release of the latent heat of fusion, then the tubes were transferred to evacuated Dewar's filled with ethanol and surrounded by liquid N_2 for cooling at 0.25°C/min. When the tissue reached -75°C it was plunged into liquid N_2 (-196°C) for storage.

Thawing Procedure (Fig. 3)

Tissue was thawed by standing well separated in racks in room air (3.5°C/min). Supernatant was aspirated after centrifugation (450 G) and 5 ml of 0.75 M Sucrose in the Medium 199 solution was added per ml of initial tissue frozen (2.5 ml per 0.5 ml pancreatic fragments in each tube) to remove remaining intracellular Me_2SO from the pancreatic fragments. This solution was agitated at 0°C for 30 min. The sucrose was diluted serially at 25°C in 5 minute steps with 1, 1, 2, and 4 volumes of Medium 199 solution while shaking at 22°C. The mixture was centrifuged, the pellet washed, spun, and resuspended to 30 ml with Medium 199 solution for transplanting.

Transplantation

After redraping in acutely transplanted animals or after reopening the abdomen in delayed transplants, the spleen was delivered through the wound and onto the abdominal wall. Two hilar splenic veins were isolated and cannulated with PE90 tubing. The splenic pedicle was clamped and the islet containing solution refluxed up the veins and into the sinusoidal spaces by slow hand injection. The clamps were removed

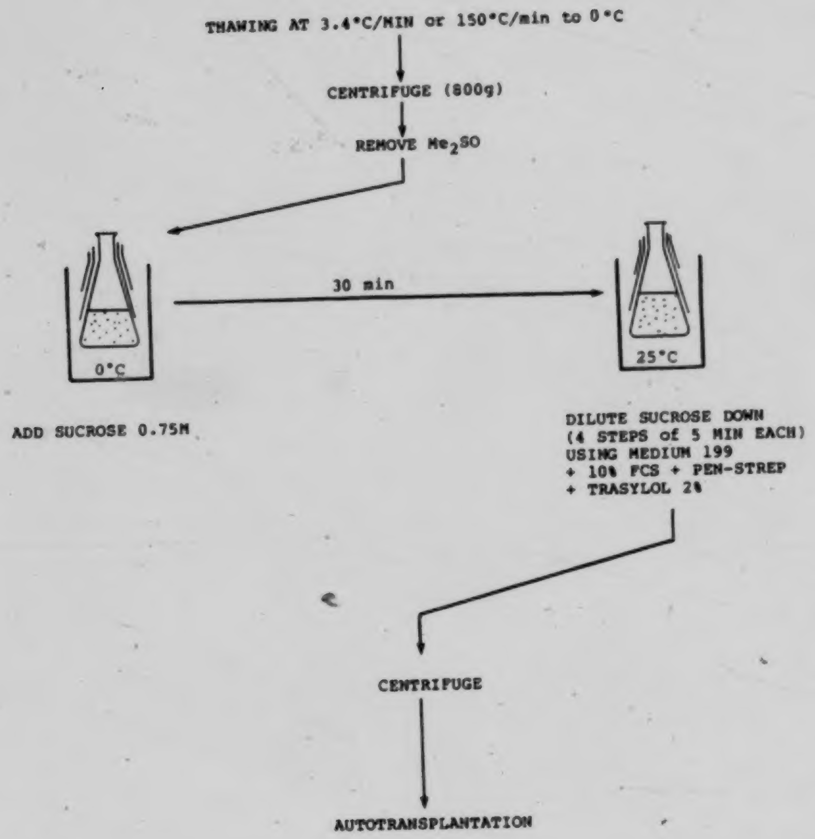
THAWING AT 3.4°C/MIN or 150°C/min to 0°C
CENTRIFUGE (800g)
REMOVE Me_2SO
30 min
0°C
25°C
ADD SUCROSE 0.75M
DILUTE SUCROSE DOWN
(4 STEPS of 5 MIN EACH)
USING MEDIUM 199
+ 10% FCS + PEN-STREP
+ TRASYLOL 2%
CENTRIFUGE
AUTOTRANSPLANTATION

FIGURE 3. THAW PROCEDURE.

from the splenic pedicle 5 minutes after reflux, the spleen returned to the abdomen and the wound closed.

For portal infusion, a mesenteric venous radical was isolated and cannulated with PE90 tubing which was advanced to the junction of splenic and superior mesenteric veins. A 3-way stopcock was attached to a manometer to allow monitoring of portal venous pressure. The islet solution was brought to total 60 ml after addition of 100,000 units of aprotinin and 600 units of heparin. Infusion was by 5 ml aliquots over 5 minute periods interspersed by waiting 5-10 minutes for portal pressure to stabilize. Infusion lasted about 2 hours.

Experimental Design

A. Control Groups

1. Apancreatic Controls (n=7)

Observation followed pancreatectomy in these animals. Sacrifice was performed when weight loss exceeded 20% of body weight or weakness prevented normal ambulation.

2. Acute Autotransplantation (n=6)

The dispersed pancreatic fragment solution was autotransplanted by splenic venous reflux immediately following tissue processing.

B. Alternate Transplantation Sites

3. Portal Infusion (n=2)

The islet containing fragments were transplanted into the portal venous system via cannualtion of a mesenteric venous radical.

C. Studies of Allotransplantation and Immunosuppression

4. Acute Allotransplantation (n=6)

Fresh allografts were performed and no immunosuppression administered.

5. Acute Allotransplantation with Azathioprine and Prednisone (n=6)

Azathioprine (Imuran, Wellcome) 3 mg/kg and prednisone 2 mg/kg were administered orally the day prior to surgery and from the second postoperative day onwards. Intravenous azathioprine and methylprednisolone sodium succinate (Solumedrol, Upjohn) in the same dosages were given on the day of surgery and the first postoperative day.

6. Acute Allotransplantation with Cyclosporin A (n=8)

a. CsA by month

2 animals received CsA oral drinking solution (Sandimmune, Sandoz Ltd., Basel, Switzerland) 25 mg/kg (in olive oil) once daily beginning 1 day prior to transplant.

b. CsA parenterally, then orally

6 animals received CsA oral drinking solution 25 mg/kg p.o. the day prior to surgery and from the second postransplant day onwards. Perioperatively, CsA solubilized in medium chain triglyceride oil (MCT Oil, Mead Johnson, Belleville, Ontario) and absolute ethanol was administered 12.5 mg/kg twice daily by intravenous bolus injection.

D. Studies of Islet Storage

7. Autotransplantation following Cyropreservation (n=12)

12 dogs received autotransplants of islet bearing pancreatic fragments after cryopreservation storage for 1 to 3 days.

8. Allotransplantation following Cryopreservation (n=6)

Allotransplantation followed storage in liquid N_2 for 1 to 2 days.

Biochemical Analysis

Plasma Sampling:

1. Fasting plasma levels of glucose and insulin were obtained preoperatively, then daily for 3-7 days postoperatively, weekly thereafter.
   In allotransplanted animals, rejection was defined as the day of onset of permanent hyperglycemia with a blood glucose of > 150 mg/dl.
2. Serum glutamic oxalacetic transaminase (SGOT), alkaline phosphatase (alk phos), creatinine and bilirubin were obtained preoperatively and 1-2 times weekly postop.
3 Intravenous glucose tolerance tests (IV GTT) using 0.5 mg/kg glucose were performed preoperatively and on a monthly basis postoperatively. K values (percent decline blood glucose per minute) were determined from the 5,10,15 and 30 min samples by the method of Moorehouse(260). A nondiabetic K value is > 1.0% per minute decline in blood glucose.
4. Serum levels of CsA were determined daily from the onset of

administration for the first 2 weeks, then 3 times weekly.

Analysis:

1. Blood glucose was measured by the glucose oxidase method on a Beckman glucose analyser.
2. Plasma immunoreactive insulin (IRI) was determined by double-antibody radioimmunoassay using porcine insulin standards (Wellcome Reagents Ltd.)(261).
3. SGOT, alk phos, bilirubin and Creatinine were measured by enzymatic methods on a Beckman Trace III analyzer.
4. Serum CsA was measured by single antibody method using test kits supplied by Sandoz (Cyclosporin RIA-kit).

Tissue

Samples of raw pancreas, post isolation and post cryopreservation tissue were fixed in Bouin's fixative. Sections of transplanted spleen and liver were fixed in 10% formalin. Immunocytochemical staining was performed through the courtesy of Dr. L. Jewell(262).

Samples of other tissues obtained at necropsy were fixed in 10% formalin and stained with hematoxylin and eosin.

Statistical Analysis

All results were expressed as mean ± SEM unless otherwise stated. Analyses were with Student's t test for unpaired data.

V **RESULTS**

A. Control Groups

1. Apancreatic Controls (n=7)

One animal died of a duodenal perforation on the fourth postoperative day and was excluded from further study. Plasma glucose levels reached 351±14 mg/dl by the first postoperative day and remained elevated until death at 6.0±0.5 days (Fig. 4, Table 1). Peripheral plasma insulin levels remained below assay sensitivity (1 m unit/L) throughout follow-up (Fig. 5). K values 3 days postoperatively were 0.61±0.13% (n=3), compared to 2.61±0.33% preoperatively (Table 2). All animals lost weight steadily from pancreatectomy to death, loss was 0.53±0.12 kg daily (Table 3).

Autopsy confirmed the complete absence of residual *in situ* pancreatic tissue in this group and all other animals in this study.

2. Acute Intrasplenic Autotransplantation (n=6)

All freshly autotransplanted animals were normoglycemic on day 1 post-op, mean plasma glucose was 97±4.4 mg/dl. After a mild (<140 mg/dl) transient (0-10 days) rise in plasma glucose all remained normoglycemic for >15 months (Fig. 4). Fasting peripheral plasma insulin levels remained unchanged from pre-op levels for 3 weeks after transplantation then fell to approximately half-pre-op values. They subsequently climbed back to values not significantly different from preoperative values by 12 months follow up and remained so until > 18

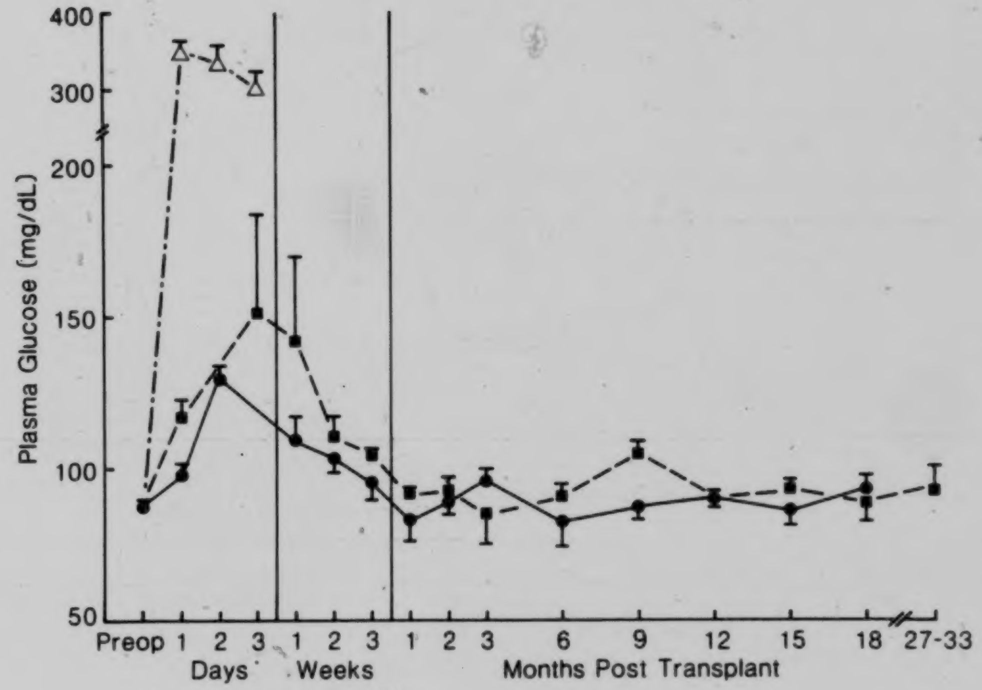

FIGURE 4. FASTING PLASMA GLUCOSE:

apancreatic control, n=6 (△— · — △);
acute autotransplant, n=6 to 3 months, n=5 at 4-14 months, n=4 thereafter (●———●);
autotransplant post cryopreservation, (Group 7) n=4 to 18 months, n=2 at 27-33 months (■– – – –■).

Table 1: Metabolic and Survival Data
(Mean ± SEM)

Group (n=6 for all)	Blood Glucose day 1 (mg/dl)	Days to Hyperglycemia	Insulin production 2m U/L (days)	Survival (days)
Apancreatic control	324,350,420,320,366,312 mean 351±14.0	1	0	5,8,7,5,5,6 mean 6.0±0.5
Autotransplant	108,104,107,93,93,80 mean 97±4.4	540(4),450(1)	>540(4),450(1)	>540(4),450(1),120(1)
Allotransplant: Non immunosuppressed	84,58,56,76,120,112 mean 84±11.0	4,4,4,5,8,5 mean 5.0±0.6	9,9,8,5,8,5 mean 7.3±0.76	11,10,25,12,28,10 mean 16.0±3.4
Allotransplant: Aza/Pred	98,117,134,164,155,122 mean 132±10.0	3,2,2,1,1,2 mean 1.8±0.3	10,7,7,7,8,8 mean 8.2±0.34	18,13,14,8,13,15 mean 13.5±1.4
Allotransplant: CsA	107,105,117,98,104,101 mean 105±2.7	30,41,17,14,10,4 mean 19.3±5.6	34,41,20,14,10,7 mean 21.0±5.6	44,50,30,30,23,23 mean 33.3±4.6
Cryopreserved Allotransplant	197,129,118,154,150,170 mean 154±11.6	1,4,6,1,12,1 mean 4.2±1.8	7,4,32,6,11,11 mean 11.8±4.2	15,18,58,18,18,15 mean 23.7±6.9
Cryopreserved Autotransplant (n=4)	110,112,112,135 mean 117±5.9	>450	>450	>450

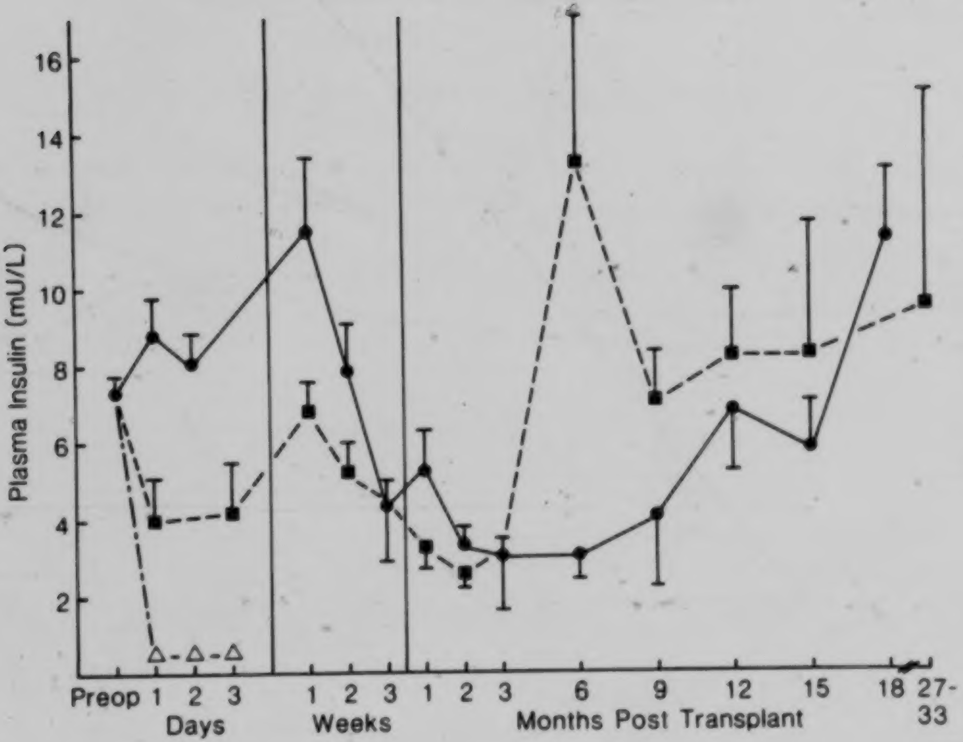

FIGURE 5. FASTING PLASMA INSULIN:

apancreatic control, n=6 (△— · —△);
acute autotransplant, n=6 to 3 months, n=5 at 4-14 months, n=4 thereafter (●———●);
autotransplant post cryopreservation, (Group 7) n=4 to 18 months, n=2 at 27-33 months (■– – – – ■).

Table 2: Intravenous Glucose Tolerance Test K Values
(% decline glucose/min; Mean ± SEM)

Group	Pre-op	Postop	Time
Non-operated Control (n=66)	3.26±0.13		
Apancreatic Control (n=6)	2.61±0.33	0.61±0.13 (n=3)	3 days
Autotransplant (n=6)	2.84±0.64	1.30±0.12	1 month
		1.67±0.12	3 "
		1.55±0.25*	6 "
		1.10±0.26	12 "
		1.51±0.24**	18 "
Cryopreserved Autotransplant (n=4)	4.07±0.66	1.59±0.17	1 month
		2.06±0.08	2 "
		2.09±0.32	3 "
		1.66±0.32	6 "
		1.56±0.26	12 "
		1.51(n=1)	27 "
Allotransplant: Non immunosuppressed (n=6)	3.36±0.44	---	---
Allotransplant: Aza/Pred (n=6)	3.36±0.51	---	---
Allotransplant: CsA	3.06±0.34	1.47±0.21 (n=2)	3 weeks

*n=4, 1 death at 4 months due to bowel obstruction, 1 value unavailable
*n=4, second animal sacrificed at 15 months (diabetic)

Table 3: Early Postransplant Weight Loss

(Mean ± SEM)

Group (n=6, all groups)	Preop wt. (kg)	Wt. Loss, 4 days (kg)	Wt. Loss, 9 days (kg)	Rate of Wt. Loss, First 4 days (kg/day)	Rate of Wt. Loss First 9 days (kg/day)
Apancreatic control	19.7±1.0	1.70±0.3	0.53±0.12		
Autotransplant	19.6±1.1	0.70±0.16	0.95±0.18	0.18±0.04	0.11±0.02
Cryopreserved Autotransplant (n=4)	20.6±0.7	1.00±0.30	1.18±0.30	0.25±0.14	0.13±0.04
Allotransplant Non immunosuppressed	20.2±1.0	0.97±0.20	2.30±0.26	0.24±0.05	0.26±0.03
Allotransplant Aza/Pred	23.9±0.8	3.98±0.45	6.67±0.53	1.00±0.11	0.79±0.07
Allotransplant CsA	21.4±0.7	1.22±0.12	1.78±0.32	0.31±0.03	0.20±0.04

months (Fig. 5). Five of 6 dogs survived over 15 months with the single early death being due to a bowel obstruction at 4 months post transplant. One animal began losing weight rapidly at 14 months, was diabetic on IV GTT at 15 months (K=0.31) and was sacrificed. The remaining 4 animals were sacrificed following their use in metabolic testing at 20 months post transplant. All 4 were normoglycemic with normal insulin levels at the time of sacrifice.

K values on intravenous glucose tolerance testing were all in the non diabetic range at all periods to 9 months although reduced from preop values (Tables 2,4). One animal began showing diabetic K values on IV GTT at 9 months although remaining normoglycemic and surviving to 18 months. The remaining 3 animals had nondiabetic K values for > 18 months (throughout follow up) (Fig.8, Tables 2,4). Peripheral insulin levels rose following glucose injection but not to the degree seen preoperatively (Fig. 9, Table 5).

All animals lost weight for the initial 1 to 2 months post transplant, mean loss being 2.57±0.62 kg, or about 13% of body weight. Subsequently, 1 animal continued to lose weight slowly (0.5 kg/mo); 2 animals lost weight up to 5 months post-op, then stabilized and began slow regaining weight, and the remaining 2 weighed more at sacrifice than preoperatively, mean weight change was -1.3 ± 2.7 kg (Table 3,7; Fig. 6, 7). Dog C454 weighed 2.9 kg more at 10 months than preoperatively, this being due to growth in body size of this adolescent dog, not simply increase in adipose tissue.

Table 4: Intravenous Glucose Tolerance Test
(plasma glucose, mg/dl, mean ± SEM)

Group	Time 0	1	5	10	15	30	60	90
Preoperative Control (n=66)	86.2±1.4	606.4±48.5	263.9±4.8	218.7±3.7	187.8±3.4	119.1±3.8	94.6±1.8	89.2±1.4
Autotransplant (n=6)								
1 month	86.5±6.4	625.8±78.4	248.0±11.9	229.5±9.0	212.7±7.4	178.7±8.7	135.5±9.9	112.5±7.4
3 months	96.3±3.5	603.2±104.5	284.2±23.4	238.2±14.4	219.2±15.6	183.0±14.5	139.0±13.7	118.7±13.1
6 months*	85.2±11.3	750.5±118.7	280.8±27.6	241.0±16.4	229.0±21.3	187.8±20.9	166.0±17.1	140.8±17.4
12 months	90.0±2.8	788.0±92.9	257.0±14.8	229.0±12.0	213.4±14.2	192.0±14.0	147.8±12.0	126.8±8.4
18 months**	91.2±2.7	558.2±95.3	262.2±10.3	234.8±11.6	210.8±9.8	181.2±15.1	140.2±18.6	120.5±16.2
Cryopreserved Autotransplant (n=4)								
1 month***	91.7±2.7	714.0±184.0	276.7±9.0	243.7±8.0	222.0±8.3	183.3±9.3	131.0±9.8	108.3±6.2
3 months	85.2±9.9	661.2±202.4	295.8±41.0	227.0±22.5	208.0±20.6	163.5±12.4	120.8±9.0	96.5±6.3
6 months	87.2±2.1	516.5±119.8	231.8±18.3	153.8±43.0	187.0±12.9	149.8±5.8	120.5±3.9	107.0±2.4
12 months	85.8±2.1	595.8±150.1	247.0±22.0	219.5±10.8	208.2±19.1	165.8±14.3	128.8±11.1	109.2±7.8

* n=4 1 death at 4 months due to bowel obstruction, 1 unavailable

** n=4 second animal sacrificed at 15 months, emaciation, diabetic

*** n=3 1 animal unavailable

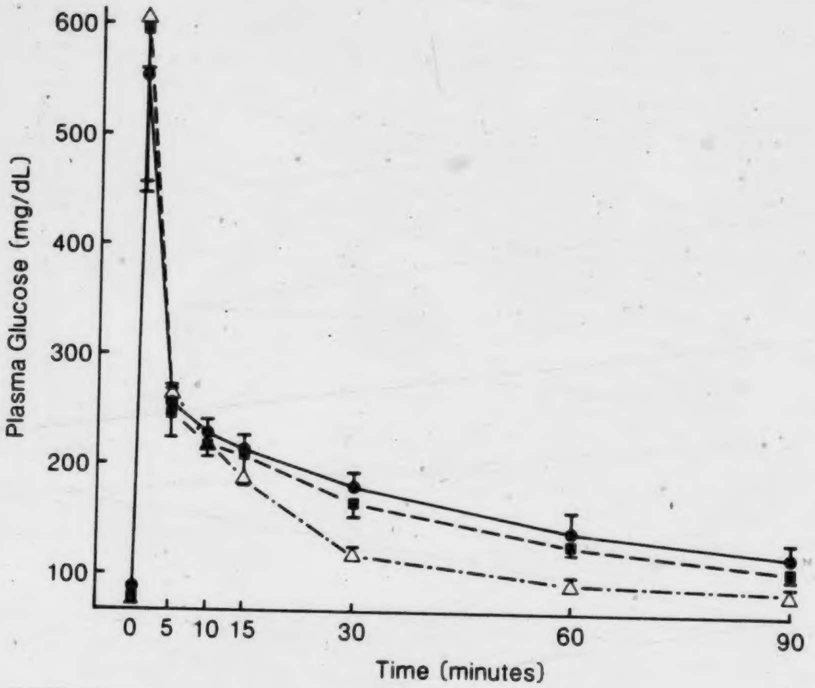

FIGURE 8. INTRAVENOUS GLUCOSE TOLERANCE TEST, PLASMA GLUCOSE:

preoperative control, n=66 (△— - — - △);
acute autotransplant 18 month follow-up, n=4 (●————●);
autotransplant post cryopreservation (Group 7) 12 month follow-up, n=4 (■- - - -■).

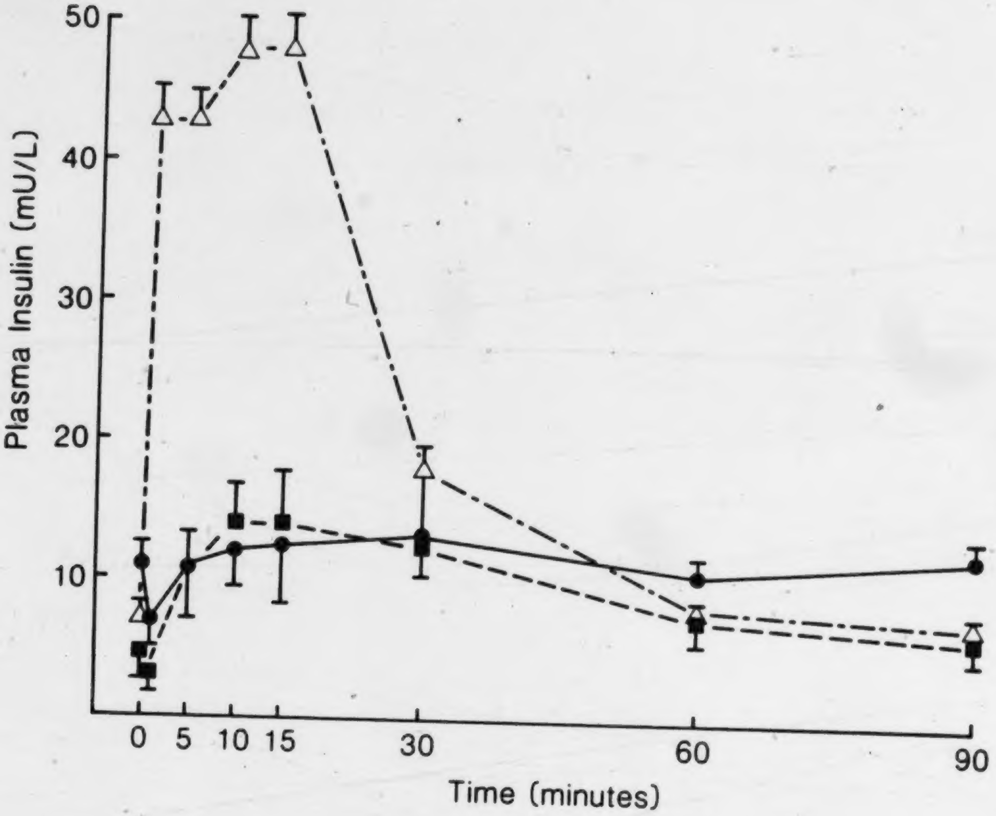

FIGURE 9. INTRAVENOUS GLUCOSE TOLERANCE TEST, PLASMA INSULIN:

preoperative control, n=66 (△— · —△);
acute autotransplantation 18 month follow-up, n=4 (●————●);
autotransplant post cryopreservation (Group 7) 12 month follow-up, n=4 (■– – – – ■).

Table 5: Intravenous Glucose Tolerance Test Insulin Response
(mU/L, mean ± SEM)

Group	N	Time (min.) 0	1	5	10	15	30	60	90
Preoperative Control	66	7.5±0.7	42.9±2.6	43.2±2.3	48.1±2.5	48.4±2.6	18.1±1.5	8.2±0.6	7.3±0.6
Autotransplant									
1 month	6	4.2±0.8	3.6±1.5	10.2±2.4	8.8±2.2	8.0±1.9	6.3±1.2	3.8±0.6	3.8±0.5
3 months	6	3.0±1.4	3.3±1.6	7.2±2.6	7.7±2.8	6.0±2.6	5.7±2.5	5.2±2.0	3.7±1.5
6 months	4*	2.2±0.6	2.2±0.6	6.5±1.0	5.8±1.1	5.0±0.9	5.0±1.3	4.2±1.8	3.2±1.1
12 months	5	5.8±1.4	3.4±1.4	6.6±2.4	5.2±2.1	5.2±1.6	4.2±3.0	3.0±2.3	4.2±1.6
18 months	4**	11.0±1.7	7.0±2.0	10.8±2.8	12.0±2.7	12.5±4.2	13.5±4.7	10.8±1.4	12.2±1.5
Cryopreserved Autotransplant									
1 month	3***	3.7±0.3	4.0±1.2	15.0±2.6	16.3±1.4	15.0±1.5	11.7±0.7	6.0±2.1	4.3±0.7
3 months	4	3.2±0.6	4.0±1.5	11.2±3.2	12.8±3.4	12.0±3.1	8.0±2.4	3.0±0.6	2.2±0.8
6 months	4	13.2±3.9	6.8±3.2	10.2±3.1	17.0±6.6	14.5±4.8	8.2±4.2	6.5±4.0	9.0±3.9
12 months	4	4.5±2.0	3.2±1.6	10.8±3.6	14.2±3.0	14.0±3.8	12.0±1.9	7.5±1.8	6.2±1.7

* n=4: 1 death at 4 months (bowel obstruction), 1 value unavailable

** n=4: second animal sacrificed at 15 months, diabetic K value, weight loss

** n=3: 1 value unavailable

Table 7: Long Term Weight Change

Autotransplant

dog	months post transplant preop	1	2	3	5	7	8	9	10	12	15
C229	18.0	15.6	13.3	12.5	12.3	12.7	12.7	12.5	12.6	12.8	12.6
C363	21.6	18.9	18.5	18.6	+						
C162	22.9	22.6	19.6	19.4	19.6	19.0	19.6	18.6	19.6	18.4	17.0
C386	20.7	20.0	19.2	19.8	19.7	20.4	20.4	20.6	21.2	20.9	18.5
C454	19.0	18.5	18.6	19.3	20.2	20.8	21.1	24.1	21.9	22.2	23.0
C460	15.2	13.2	13.0	13.2	14.1	13.4	13.2	14.8	13.6	13.4	13.8
mean	19.6	16.1	17.0	17.1	17.2	17.3	17.4	18.1	17.8	17.5	17.0
SEM	1.1	1.4	1.2	1.4	1.6	1.8	1.8	2.1	2.0	1.9	1.8

Cryopreserved Autotransplant

dog	months post transplant preop	1	4	5	6	7	8	9	12	13
C552	19.8	17.8	19.4	19.4	19.1	19.3	22.1	20.4	20.8	20.2
C588	21.2	19.0	21.2	22.6	21.0	21.8	24.0	21.9	22.4	22.5
C589	22.3	19.8	19.2	18.9	19.2	21.2	19.1	19.2	19.0	19.5
C612	20.0	15.8	15.2	15.0	15.5	16.9	15.0	14.9	15.0	14.8
mean	20.8	18.1	18.8	19.0	18.7	19.8	20.0	19.1	19.3	19.2
SEM	0.6	0.9	1.3	1.6	1.2	1.1	2.0	1.5	1.6	1.6

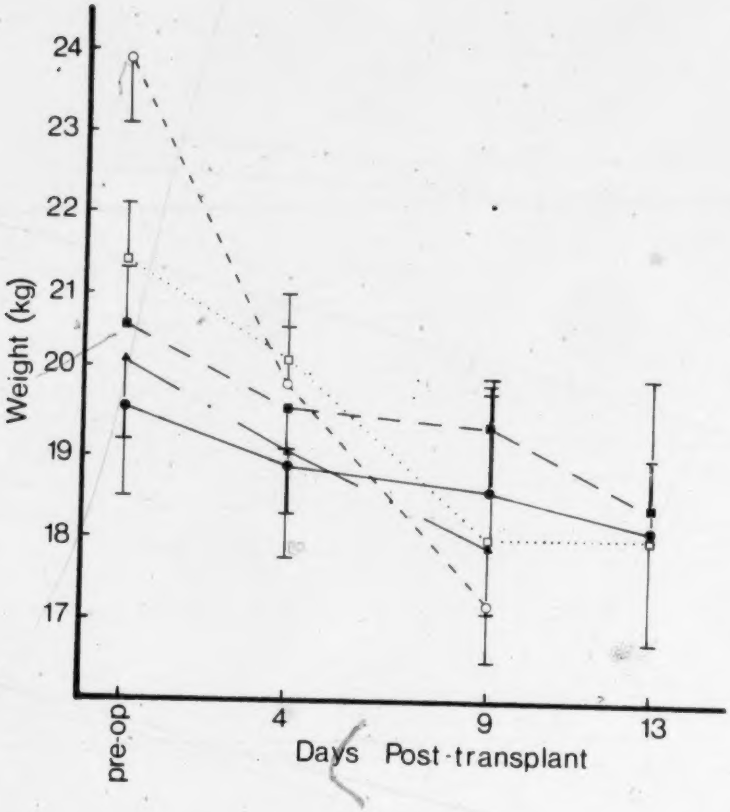

FIGURE 6. EARLY POST-TRANSPLANT WEIGHT LOSS:

acute autotransplant, n=6 (●————●);
autotransplant post cryopreservation, n=4 (■— — —■);
nonimmunosuppressed allotransplant, n=6 (▲ ——·—— ▲);
allotransplant with azathioprine - prednisone, n=6 (○– – – – –○);
allotransplant with cyclosporin A, n=6 (□·······□).

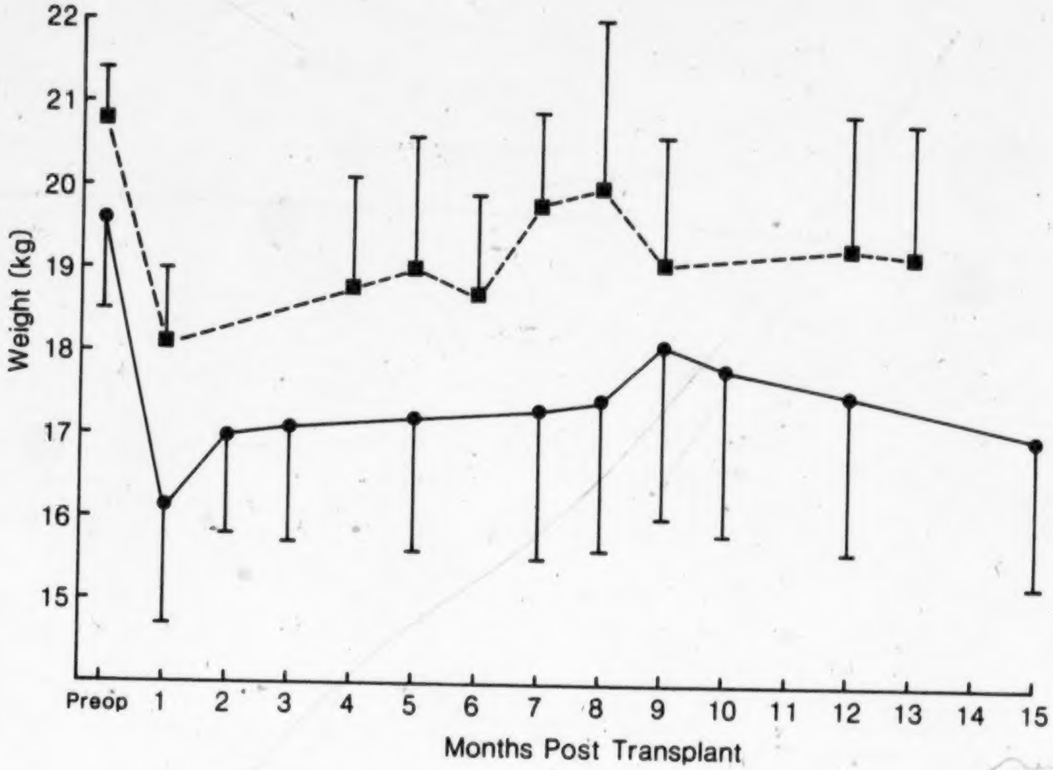

FIGURE 7. LONG TERM WEIGHT CHANGE:

acute autotransplant, n=6 (●———●);
autotransplant post cryopreservation, n=4 (■- - - - -■)

B. Alternate Transplantation Sites

3. Intraportal Autotransplantation (n=2)

In the first animal, the entire pellet (17 ml) was transplanted in 55 ml total suspension over 65 minutes. A gradual but steady increase in portal pressure occurred from a baseline of 4.5 cm H_2O, reaching a maximum pressure of 33 cm water and falling to 19 cm water within 40 minutes of the end of infusion. This animal was normoglycemic for 2 days, became hyperglycemic (plasma glucose 166 mg/dl) on day 3 and by day 6 the plasma glucose had reached 512 mg/dl. Peripheral fasting plasma insulin levels were normal for 6 days, but then dropped gradually to insignificant levels by 3 weeks post-transplant. The animal was sacrificed at day 37.

In the second dog the portal pressure had reached 39.5 cm water when only half the 20 ml of pellet had been infused (30 ml of total 60 ml solution). Despite slowing the infusion and stopping intermittently, only 75% of the graft could be infused. The last 5 ml aliquout caused an abrupt rise in portal pressure to 56 cm water which had only decreased to 47 cm at closure 20 minutes later. This animal died 12 hours post-operatively and at autopsy was found to have total portal veinous thrombosis.

C. Studies of Allotransplantation and Immunosuppression

4. Allotransplantation without Immunosuppression (n=6)

Normoglycemia was achieved in all 6 animals post-operatively (mean 84±11 mg/dl) with plasma glucose results paralleling autotransplanted animals for the first 3 days (Fig. 10). Rejection occurred at 5.0±0.6 days post-transplant and survival was 16.0±3.4 days (Table 1). Peripheral plasma insulin levels remained within normal limits for 4 days, then plummetted with rejection on day 5 (Fig. 11). Nonimmunosuppressed allotransplanted dogs lost weight at a rate of 0.24 kg/day for the initial 4 post operative days (full diet restored on day 4), and at a rate of 0.27 kg/day from days 5 to 9 when feedings had been restored (but rejection had occurred in most animals). Mean weight loss was 0.26±0.03 kg/day over the first 9 postoperative days (Table 3, Figure 6).

The first 2 animals in this group suffered episodes of tachycardia, hypotension and respiratory arrest shortly after receiving their splenic venous reflux grafts. Both were resuscitated successfully with parenteral fluids and artificial ventilation. Subsequent dogs were preloaded with 1000-1500 ml Ringer's lactate intravenously, prior to transplantation, and no further difficulties were encountered.

5. Allotransplantation with Azathioprine-Prednisone Immunosuppression

Four of 6 animals were normoglycemic the first postoperative day, the other 2 had plasma glucose levels of 155 and 164 mg/dl. Hyperglycemia occurred 1.8±0.3 days after transplantation and survival was 13.5±1.4 days (Fig. 12,13, Table 1). Peripheral plasma insulin levels fell to abnormally low levels (<2 mU/L) 8.2±0.6 days post transplant, 6.5 days after hyperglycemia had occurred. Weight loss averaged 0.79±0.07 kg/day over the first 9 post operative days, being 1.0 kg/day during the initial 4 days, than slowing to 0.61 kg/day for the next 5 (Table 3, Fig. 6).

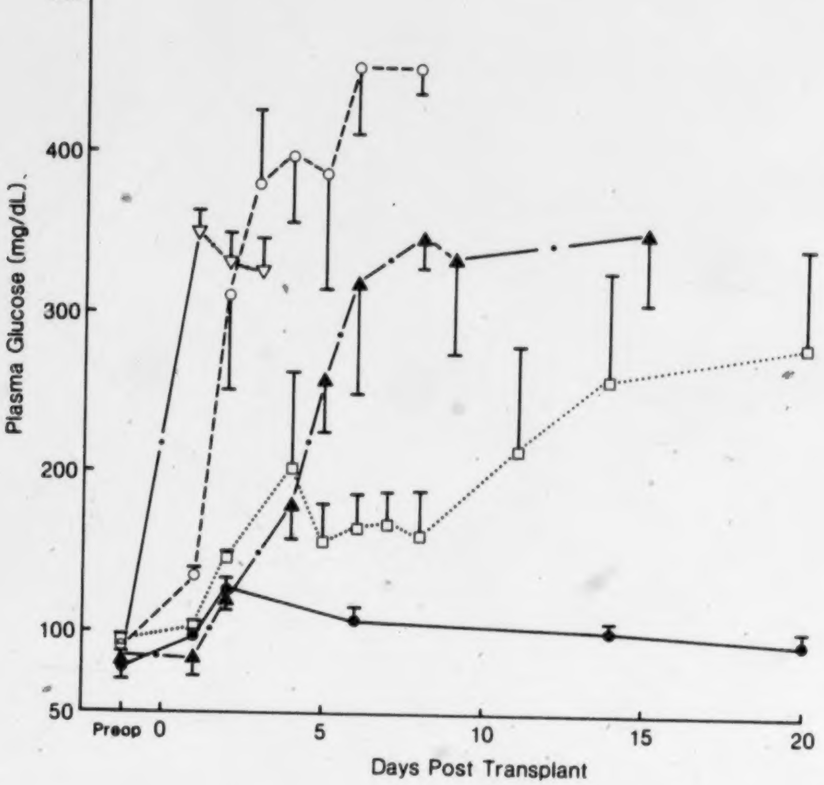

FIGURE 10: POST TRANSPLANT PLASMA GLUCOSE:

apancreatic control, n=6 (▽ —— · —— ▽);
acute autotransplant, n=6 (●————●);
nonimmunosuppressed allotransplant, n=6 (▲—— · —— ▲);
allotransplant with azathioprine - prednisone, n=6 (○- - - - - -○);
allotransplant with cyclosporin A, n=6 (□·········□).

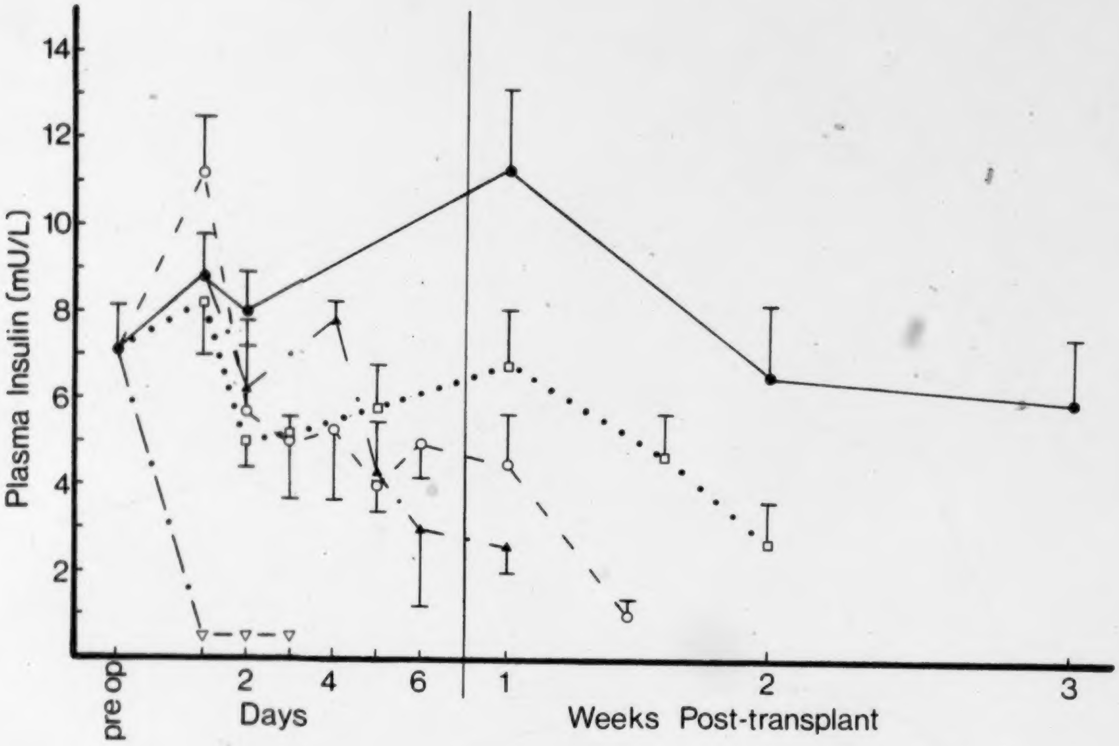

FIGURE 11. POST TRANSPLANT PLASMA INSULIN:

apancreatic control, n=6 (▽— · — ▽);
acute autotransplant, n=6 (●——●);
nonimmunosuppressed allotransplant, n=6 (▲ — · — ▲);
allotransplant with azathioprine - prednisone, n=6 (○– – – –○);
allotransplant with cyclosporin A, n=6 (□· · · · · · □).

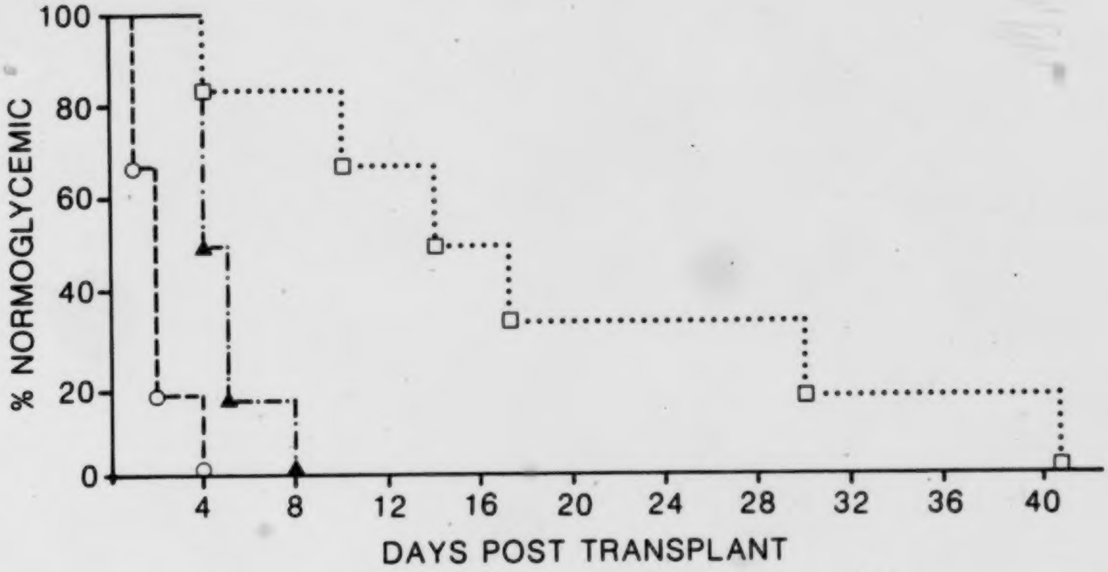

FIGURE 12. ALLOTRANSPLANT, DURATION OF NORMOGLYCEMIA:

nonimmunosuppressed allotransplant, n=6 (▲—— · ——▲);
allotransplant with azathioprine - prednisone, n=6 (○— — — —○);
allotransplant with cyclosporin A, n=6 (□········□).

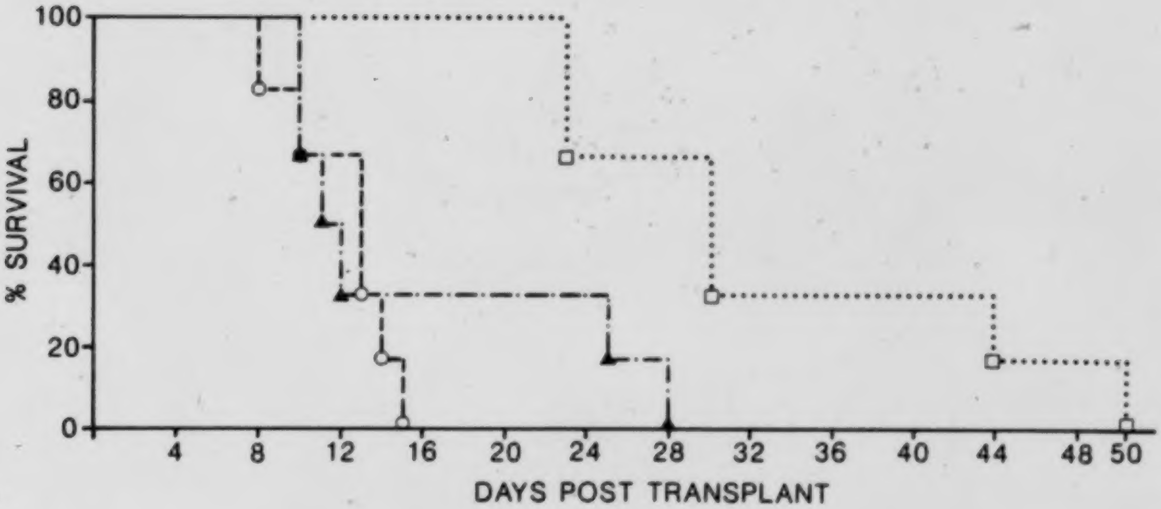

FIGURE 13. ALLOTRANSPLANT, SURVIVAL:

nonimmunosuppressed allotransplant, n=6 (▲— · —▲);
allotransplant with azathioprine - prednisone, n=6 (○– – – –○);
allotransplant with cyclosporin A, n=6 (□·······□).

One animal died of pneumonia 8 days post transplant. Three dogs developed wound infections and another a superficial wound dehiscence. None had cleared before death.

6. Allotransplantation with Cyclosporin A (CsA) Immunosuppression

a. Pilot Group - oral CsA (n=2)

The first animal rejected on the third postoperative day, secreted insulin in measurable amounts for 9 days, and was sacrificed at 26 days. An unsuspected purulent wound infection was found at autopsy. The second dog rejected on the twelfth day after transplantation and ceased producing measureable amounts of insulin on day 13. He was sacrificed at 33 days.

b. Oral and Parenteral CsA (n=6)

Mean plasma glucose on the first post ransplant day was 105±2.7 mg/dl. All dogs were normoglycemic for the first 3 days post transplant. Plasma glucose levels then rose for periods from 5 to 11 days with 5 of 6 dogs becoming frankly hyperglycemic (b.g > 150 mg/dl) (Fig. 14). One animal did not return to normoglycemia before rejecting his graft, but the remaining 5 dogs all returned to normal plasma glucose levels for variable times before ultimately rejecting. The onset of permanent hyperglycemia (rejection) was at 19.3±5.6 days post transplant and survival was 33.3±4.6 days (Table 1, Fig. 12,13). Peripheral plasma insulin levels dropped abruptly within 1 to 2 days of the onset of rejection as gauged by hyperglycemia (Table 1). IV GTT's were obtained on the 2 animals who remained normoglycemic at 3 weeks following transplant. K values averaged 1.47±0.21% (Table 2). Mean

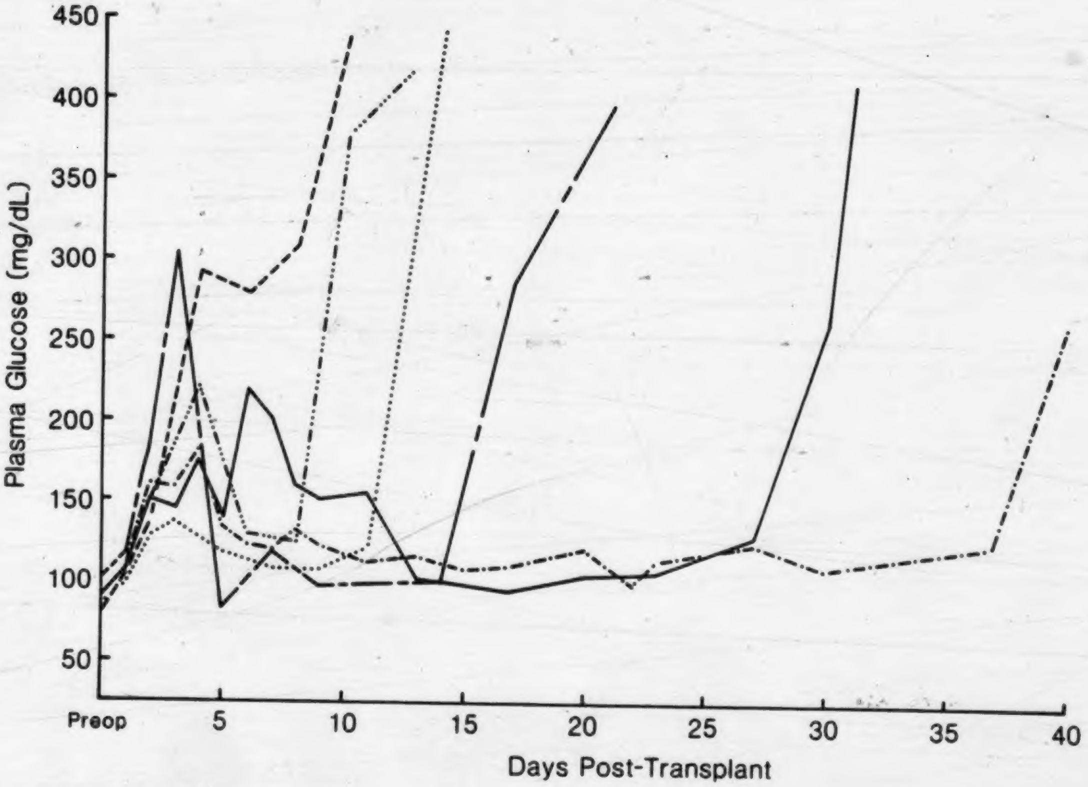

FIGURE 14. FASTING PLASMA GLUCOSE: ALLOTRANSPLANTS WITH CYCLOSPORIN A.

serum CsA levels for the first 7 days then for the remaining normoglycemic dogs up to 41 days post transplant are shown in Table 6.

Dogs in this group lost 0.30 kg/day up to the resumption of full feeding on day 4; weight loss then slowed considerably to 0.11 kg/day from days 5 to 9. Average daily weight loss over the first 9 days was 0.20±0.04 kg/day (Table 3, Fig. 6). All 5 dogs that had not rejected their grafts by the time full feeds were resumed on day 4 subsequently regained some portion of this lost weight, with gains of up to 0.5 kg over a 10 day period.

One animal developed an intussusception requiring laparotomy and small bowel resection on the second day following tranpslant. He healed his wounds rapidly, took feedings ravenously 4 days after bowel resection, gained weight and continued normoglycemic until rejection on day 17. The daily dose of CsA 25 mg/kg was never altered except for intravenous administration in the perioperative periods.

Laboratory analysis of drug toxicity

1. Elevation of serum creatinine levels was sporadic and evenly distributed through all treatment groups (Fig. 15).
2. Increase in SGOT and alkaline phosphatase were seen in most animals in the first few weeks postoperatively. Although CsA treated animals showed a tendency to higher alkaline phosphatase levels, the differences in alk phos reached statistical significance on only one day in the post operative period (Fig. 16) while levels of SGOT were never significantly different.
3. Leucocyte counts in azathioprine/prednisone treated animals showed no significant decreases during theapy.

Table 6: Serum Cyclosporin A
(mg/L)

animal number	days postop 0	1	2	3	5	7	9	11	13	17	20	23	27	31	37	41
C530	.27	.31	.72	.93	1.90	.43	.36	.23	.15	.27	.09	.10	.11	.12*		
C698	.39	.73	1.07	.69	.85	.25	.20	.26	.16	.15	.22	.59	.88	.11	.16	*
C734	.54	1.63	1.53	.22	1.08	1.25	.26	.15	.47	.28*	.20					
C646	.29	1.72	1.11	.28	.42	.42	.39	.25	*	.59						
C533	.66	1.22	2.19	.53*	.29	.43	.21		.14							
C689	.12	1.16	1.08	.73	.47	.22	.38		.22*							
mean	.38	1.13	1.28	.56	.84	.50	.30		.23							
SEM	.08	.22	.21	.11	.24	.15	.04		.05							

* rejection

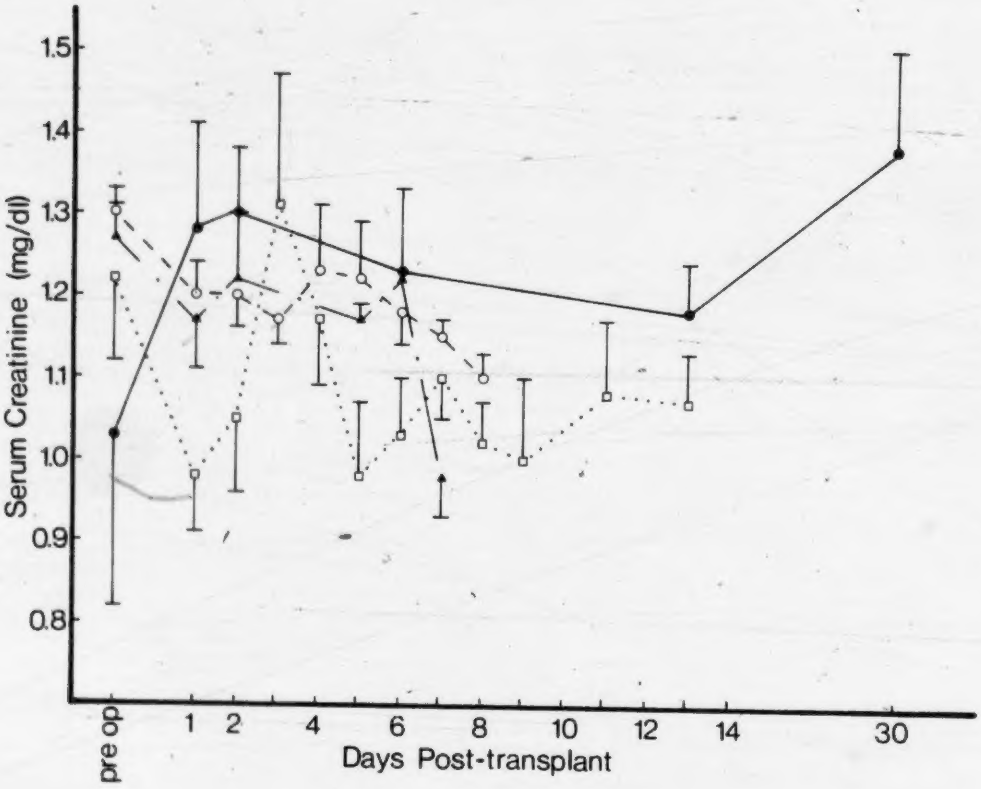

FIGURE 15. SERUM CREATININE:

fresh autotransplant, n=6 (●———●);
allotransplant without immunosuppression, n=6 (▲— · —▲);
allotransplant with azathioprine/prednisone, n=6 (○- - - - -○);
allotransplant with CsA, n=6 (□·········□).

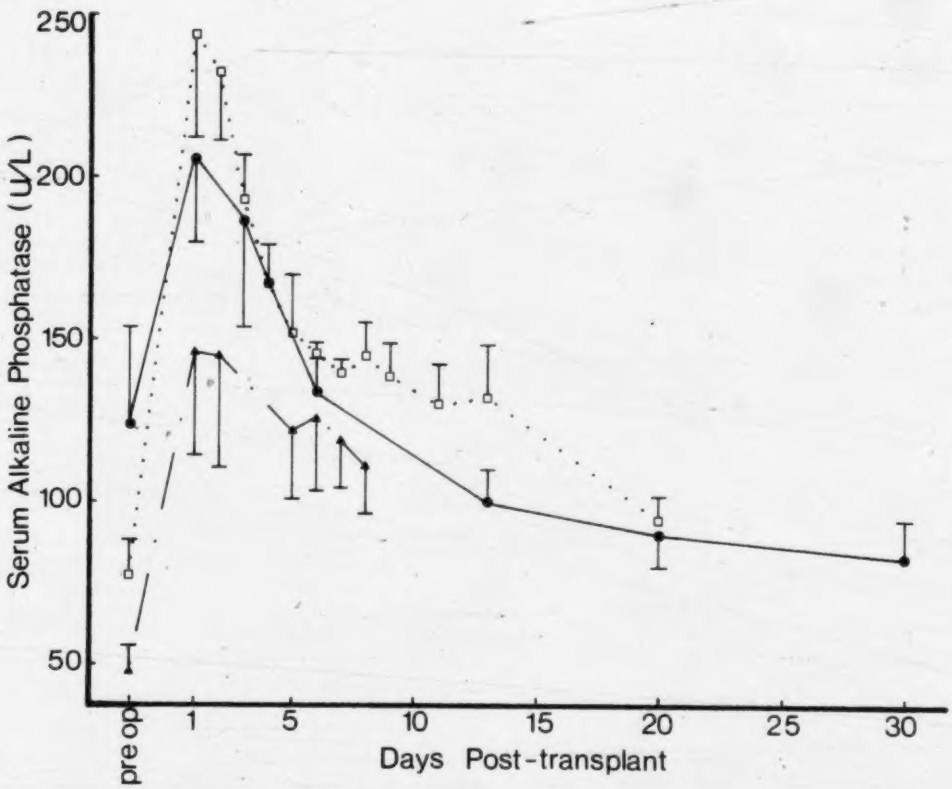

FIGURE 16. SERUM ALKALINE PHOSPHATASE:

fresh autotransplant, n=6 (●————●);
allotransplant without immunosuppression, n=6 (▲—— · —— ▲);
allotransplant with CsA, n=6 (□· · · · · · □).

D. Studies of Islet Storage by Cryopreservation

7. Autotransplantation following Cryopreservation

a. Early animals - all DMSO exposure at 25°C (n=8)

Technical errors occurred during cryopreservation in 2 animals. During Me_2SO permeation in C439, temperature increased to 40°C for 10-15 minutes; insufficient Me_2SO was added in a subsequent step and then insufficient sucrose during thawing. C479 was thawed at 2.2°C/minute. Neither animal was normoglycemic postoperatively and both were sacrificed at an ealy date. C434 was hyperglycemic immediately after transplantation, never exhibited significant fasting peripheral plasma insulin levels and died 8 days after transplant. C513 was normoglycemic for a short period following transplant but had low and steadily decreasing insulin levels leading to sacrifice on day 18. C511 was hyperglycemic the first postop day despite normal to high fasting peripheral plasma insulin levels. His plasma glucose level dropped steadily from 306 mg/dl to 222 mg/dl by 3 weeks post transplant when glucose rose dramatically and insulin levels fell, leading to emaciation and sacrifice 67 days post transplant. C427 was normoglycemic for 10 days but subsequently experienced a gradual rise in plasma glucose and a decrease in insulin levels. He was sacrificed for histological analysis while still healthy at 44 days.

Thus 4 animals of this initial group were unsuccessful and 2 were partial successes with periods of normoglycemia from 1- 7 days, borderline glucose values for 3 weeks but subsequent hyperglycemia and fall in insulin levels.

The other 2 animals in this group have been long term successes.

Initial postoperative normoglycemia was followed by a short period (1 to 4 days) of hyperglycemia, then by normoglycemia that has persisted for over 12 months. Peripheral serum insulin levels have been low in these animals and have not shown large increases with glucose infusion. K values have remained nondiabetic for 18 months in one animal that was subsequently sacrificed during metabolic studies. The second dog became diabetic at 13 months and was sacrificed.

b. Final Cryopreservation Protocol (n=4) (see page 66)

All 4 animals of the final group to receive frozen thawed islet transplants were normoglycemic on the first postoperative day, mean plasma glucose being 117±5.9 mg/dl. Thereafter, 1 animal remained normoglycemic throughout, 2 had transient (days 3 to 7) and minimal (< 140 mg/dl) elevations in plasma glucose, and the fourth was hyperglycemic for 10 days before settling to normal plasma glucose levels (Fig. 4). Fasting peripheral plasma insulin levels remained normal for 3 weeks post transplant, fell somewhat for 3 months, then returned to preop levels for the remainder of follow up (Fig. 5).

Mean weight loss by 1 month post op in these animals was 2.15 kg or 10.4% of body weight. Two animals had slow but continuing weight loss for 2 further months, then stabilized in weight. The other 2 dogs began regaining lost weight after 1 month follow up and were heavier at sacrifice than prior to transplant. Mean overall weight change at 1 year was -1.3±1.6 kg (Table 7, Fig. 7).

Three animals were sacrificed during metabolic studies at 15, 17 and 18 months post transplant (all normoglycemic), and the fourth remains alive at 27 months with a fasting blood glucose of 94 mg/dl and a K value of 1.51 (Table 2).

K values of all animals were non diabetic at 6 months with 3 of 4 non diabetic at 12 months and a mean K at 12 months of 1.56±0.26 (Table 2). Insulin response to glucose infusion was again blunted from pre op levels but not so markedly so as in acutely autotransplanted animals (Fig. 9, Table 5).

8. Allotransplantation following Cryopreservation

Three of 6 dogs were hyperglycemic the day following transplantation with plasma glucose levels between 150 mg/dl and 200 mg/dl. Fasting peripheral plasma insulin levels in all 3 were $\geq$ 2m U/L for 5 to 6 days. Sacrifice was at 14, 15 and 18 days following transplantation.

The other 3 animals were normoglycemic the day following surgery and rejected their grafts 4, 5 and 12 days after transplant. Insulin was measurable in peripheral plasma at levels $\geq$ 2m U/L for 5, 25 and 11 days respectively and survivals in these dogs were for 18, 58, and 18 days.

Mean time to hyperglycemia in the entire group of 6 dogs was 4.2±1.8 days, and in the 3 animals with a period of normal plasma glucose, rejection occurred at 7.0±2.2 . Mean period during which $\geq$ 2m U/L of insulin were present in peripheral blood was 9.5±4.0 days. Survival averaged 23.7±6.9 days (Table 1).

VI DISCUSSION

A. Control Animals

1. Apancreatic Control

The rapid onset of marked hyperglycemia and weight loss in conjunction with unrecordable peripheral plasma insulin levels and the rapid death of all apancreatic dogs graphically points out the severity of this model of diabetes. No animal in any study group was found at autopsy to have any remnant of insitu pancreas. Six animals transplanted with cryopreserved tissue were sampled between pancreatectomy and delayed transplant (following cryopreservation) and all had insulin levels below assay sensitivity ($\leq$ 1 munit/L). The reliability and severity of this model of insulin dependent diabetes mellitus makes it ideal as a control for transplant studies.

2. Intrasplenic Autotransplantation

The results confirmed the findings of Warnock et al.(80) with the reliability of this model of pancreatic dispersed fragment autotransplantation by splenic venous reflux. All 6 animals autotransplanted by this method have been normoglycemic on the first postoperative day and 4 of 5 remained normoglycemic throughout > 18 months follow up (Fig. 1). Five of 6 animals have survived for prolonged periods (> 12 months), with the only early death being due to a complication of laparotomy - a bowel obstruction due to postoperative adhesions. One dog became diabetic 13 months postoperatively and was sacrificed.

Animals transplanted using previous methods of preparation and transplantation have often required a number of days to return to normoglycemia after transplant(239). Two factors have been implicated. Enzymatic digestion, and probably high speed centrifugation as well, damages cells(240). Cellular repair must be completed before adequate carbohydrate homeostasis can be effected by the transplanted cells. Alternatively or additively, transplantation into a sinusoidal space such as occurs with intrapulp injection into the spleen could not be expected to achieve optimal metabolic results until the implanted endocrine cells could be revascularized. These new vessels would provide an efficient conduit for both presenting alterations in serum glucose levels and other influences to the endocrine cells, and for transporting secreted insulin, glucagon and other hormone products to their sites of action.

Splenic venous reflux places the endocrine cells directly in the vascular space, where hormonal products will immediately be effective in carbohydrate homeostasis. This obviates any need for exogenous insulin following transplantation, and the more optimal metabolic environment may aid in more efficient engraftment and early function(241), with less strain on endocrine cells to combat hyperglycemia in the early postoperative period.

The lack of hyperglycemia at any time following transplant also makes evaluation of alternate applications of the technique much simpler. The results of allotransplantation or of methods of storage would not be easily compared if periods of hyperglycemia could be the fault of the basic model. The reliability of the model is also advantageous in this regard.

Previous studies of pancreatic autotransplantation after total pancreatectomy have documented slow but continuing weight loss after transplant, despite normal fasting blood glucose levels(72). It has been suggested that inability of exogenous pancreatic enzyme supplements to normalize digestion was responsible(73). Our studies suggest otherwise. After an initial weight loss of about 13% body weight over 1 to 2 months, 4 of 5 surviving autotransplanted animals subsequently gained weight (Fig.7, Table 7). Indeed, 2 animals weighed more at sacrifice than they did preoperatively. It seems that a successful pancreatic dispersed fragment transplant can not only prevent weight loss, but can actually support considerable weight gain. C454 weighed 2.9 kg more at sacrifice than pretransplant, a total gain of 3.7 kg over his weight 1 month postoperatively. This pattern of weight loss over a period of 1 to 2 months followed by subsequent weight gain suggests a period of accommodation of the animal for the "neo pancreas". This may involve simply the complete vascularization of the graft, or indeed may reflect the time needed for metabolic accommodation to the lower insulin reserve available, or perhaps reflects other changes such as reinnervation of the transplanted islets rendering them more efficient in response to a glucose load. The improvement in IV GTT K values from the early postoperative period (1 month) to 3 months postop may well reflect the same phenomenon (Table 1).

One problem with this model however, has been the consistently low insulin response to glucose infusion when compared to preoperative studies (Fig. 9, Table 5). Although transplanted dogs remain normoglycemic and have shown nondiabetic K values on GTT for over 18 months, with no evidence of fatigue in 4 of 5, the metabolic performance of long term survivors has not equalled preoperative levels. GTT K values, although consistently in the nondiabetic range, have been substantially lower than preoperatively (Table 2), likely reflecting the limited reserve capacity of the grafts (as evidenced by decreased insulin secretion).

What factors may be responsible for this? Other workers have believed that islets "trapped" by surrounding exocrine tissue may not be able to function effectively. Histology has shown, however, that very few of the islets present in our preparations are "trapped" when examined in the spleen some time post-transplant (Plate 1). Long term study has shown little exocrine tissue remaining in the spleen, but it seems likely that the noxious influence of enzymes released from transplanted exocrine cells early on, could damage or destroy significant amounts of endocrine tissue. This could explain the ultimate failure at 13 months of the dispersed fragment graft in one of the autotransplanted dogs of Group 2.

It may also be that the large amount of pancreatic tissue recovered from processing (10-16 ml) may be beyond the limits of the transplant procedure to adequately handle. Since the blood supply of the spleen is largely segmental in nature, half of this large volume of tissue refluxed up a single hilar vein could result in significant disturbance of intrasplenic blood flow which may compromise the ability of endocrine tissue to survive and engraft. Histology has shown clots in intrasplenic veins that contained large amounts of infused tissue, much of which was non viable (Plate 2). An increase in purity of the product for transplantation and so a decrease in amount of tissue infused may result in a higher survival of transplanted endocrine cells and so better ultimate metabolic function. The technique of cryopreservation may have a role in this regard.

COLOURED PICTURES
Images en couleur

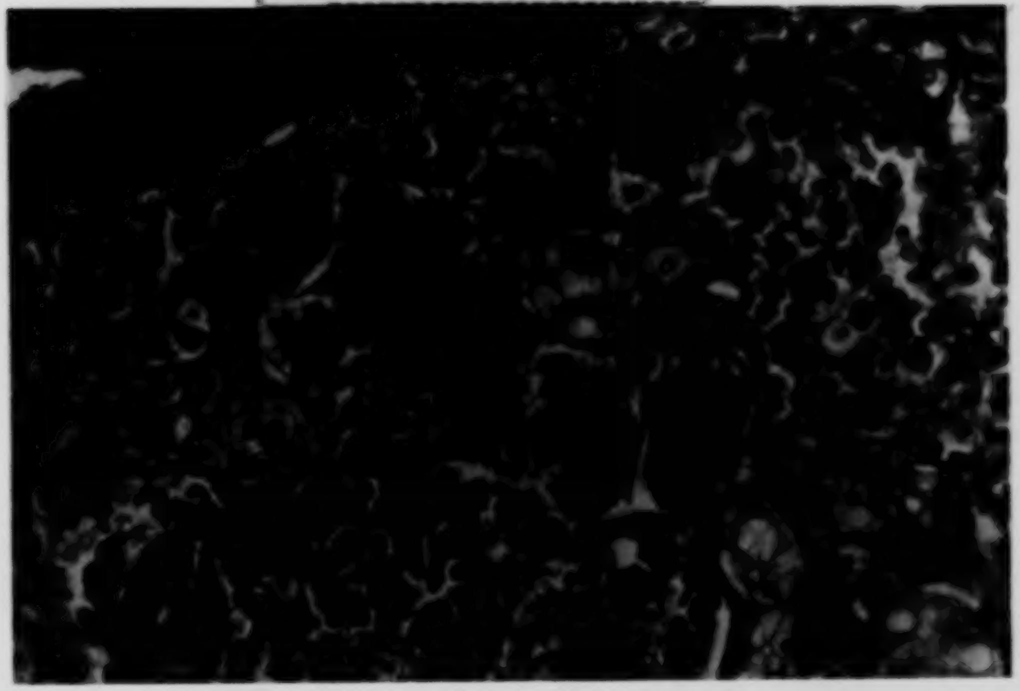

Plate 1. Intrasplenic pancreatic islet on long term follow-up: endocrine tissue (A), exocrine tissue (B), splenic pulp (C) (Gomori's aldehyde fuchsin with Mallory's trichrome counterstain)

COLOURED PICTURES
Images en couleur

Plate 2. Intrasplenic pancreatic islets: vascular space with viable islet (A) and cellular debris (B); perivascular islet (C)

The presence of the liver between the site of transplant (spleen) and the peripheral veins where insulin levels are sampled makes evaluation of insulin levels difficult. Being the major site of insulin utilization in the body, the liver can extract a large portion of insulin in portal blood on one pass through the liver. This makes statements about metabolic activity and reserve of the intrasplenic transplant somewhat tenuous when based on peripheral insulin levels. A moderate decrease in insulin secretion by the residual β cells of the intrasplenic graft could result in an increased hepatic extraction to compensate. This would result in peripheral venous insulin levels being low relative to actual insulin production by the intrasplenic graft.

The pattern of change seen in peripheral plasma insulin levels and K values supports the idea of metabolic compensation occurring with time. In groups of dogs receiving both freshly autotransplanted tissue and tissue autotransplanted after cryopreservation, insulin levels in the early postoperative period were normal or high while K values 1 month postoperatively were on average only 1.30±0.12 in acute autotransplantation and 1.59±0.17(n=3) in cryopreserved animals (Table 2). By 3 months time mean K values in the actutely autotransplanted animals had increased to 1.64±0.11 and in the frozen/thawed (FT) group had climbed to 2.09±0.32 (Table 2). At the same time fasting peripheral plasma insulin levels fell substantially (Fig. 5) as did insulin levels following glucose infusion (Table 5). In the autotransplanted group fasting plasma insulin had returned to normal levels by 18 months, as it had in the FT dogs by 12 months (Fig. 5). Insulin levels in peripheral plasma following glucose infusion had increased in both groups (Fig. 9, Table 5) but K values had dropped by 6 months and remained unchanged at

18 months and 12 months respectively for acutely autotransplanted and FT dogs (Table 2). This accommondation could be due to changes in hepatic metabolism, insulin actions at the peripheral cellular level, or to changes in the engrafted islets themselves - such as possible reinnervation. These metabolic adjustments require further investigation.

This possibility of increased hepatic extraction may explain the very low peripheral insulin levels following a glucose infusion in autotransplanted animals, when these same aimals are capable of achieving nondiabetic glucose tolerance test K values postoperatively which do not appear to show evidence of endocrine cell fatigue at up to 18 months. If the engrafted tissue was indeed only marginal in amount, as would be suggested by these low insulin levels, fatigue could be expected to occur with time. Fatigue within months has been seen by Najarian _et al_ in humans autotransplanted with marginal amounts of islet tissue after pancreatectomy for chronic pancreatitis(75).

The carboxyterminal peptide (c-peptide) that is cleaved from proinsulin, as part of the formation of metabolically active insulin, is not extracted in any significant amounts by the liver. It is produced in equimolar quantities with insulin and largely excreted unchanged by the kidney(242). Serum levels of c-peptide may thus give a much more satisfactory method of following the activities of islet transplants within the portal system. C-peptide assays of peripheral blood would be preferable to invasive sampling of splenic or portal venous blood as a method of accurately quantifying graft insulin production, especially in clinical situations.

B. Variations in Autotransplantation Protocol

3. Intraportal Autotransplantation

Transplantation of pancreatic islets to the liver has many advantages. Since the major site of insulin utilization in the body is the liver, an intraportal transplant could be expected to function more efficiently than a transplant draining to the systemic circulation(82). Theoretically an allograft to an immunologic organ such as the spleen could lead to a more severe rejection reaction than transplant to other sites. Clinically, an intraportal transplant would be technically much simpler, the spleen not being an organ particularly well suited to surgical manipulation in man. Indeed, intraportal transplantation in man could well be performed without laparotomy: by percutaneous cannulation of the portal system or by cannulation of the umbilical vein.

Unfortunately intraportal transplantation of dispersed pancreatic fragments is not without problems. Several investigators have described problems with portal hypertension of varying degrees(75,76,84) leading to patient death in at least two cases(76,92). Severe disseminated intravascular coagulation (DIC) was noted in one patient(90). Portal vein thrombosis with portal hypertension and subsequent massive upper gastrointestinal bleeding has also been seen in 1 patient(243). The long-term function of these intraportal transplants is also questionable. Of 23 patients in 3 series to undergo intraportal dispersed pancreatic fragment transplantation following 95% pancreatectomy for chronic pancreatitis, only 9 have been insulin independent indefinitely and only 1 of these had documentation of insulin production from the liver(75,76,84).

The vascular changes in the portal system have been blamed on two main factors. Large amounts of tissue thromboplastins and vasoactive amines, such as bradykinin, present in the grafts cause intense vasospasm(90,93) and intravascular thrombosis. Combined with physical disruption of flow by the introduction of relatively large amounts of tissue into the portal venous space, the obstruction to portal flow could become severe. Portal vein thrombosis has been noted to occur as the culmination of these influences(92,242) and was seen in one of the 2 dogs in my series as well.

Aprotinin is known to bind and inactivate kallikreins (including bradykinin) of man and pig, but not of dog. It has been shown to be very effective in preventing transplant induced portal hypertension in pigs, to be only partially effective in dogs, and to be ineffective in monkeys(93). Heparin, which could be expected to prevent secondary thrombosis, has had little influence in animal studies(93) but in human work at Minnesota, full heparinization was associated with lower portal vein pressures than other studies, and no instances of DIC or portal vein thrombosis(75).

Our results utilizing high doses of aprotinin (5000 KIU/kg) and fairly low doses of heparin (500 to 600 units total) are similar to those of Traverso et al with dogs(93). Moderate and reversible portal hypertension was seen in the first dog but severe portal hypertension leading to death from portal vein thrombosis occurred in the second. One could argue that full heparinization was needed but it seems evident that a much cleaner preparation of islet containing tissue will be required for safe and effective intraportal transplantation in man.

C. Studies of Allotransplantation and Immunosuppression

4. Allotransplantation without Immunosuppression

Previous studies with allotransplantation of nonpurified pancreatic fragments have demonstrated rapid rejection in both animal(143,145,159) and human trials(73). Utilizing methods that require a period of several days before normoglycemia is achieved even in autotransplant situations, some investigators have not been able to achieve normoglycemia before rejection occurred(160,236). Studies of methods to delay rejection of allografts would obviously be difficult with such methods of transplantation. Sutherland has suggested a period of 2 days of normoglycemia following islet allografting to verify successful engraftment and function(240). Subsequent hyperglycemia can then be confidently ascribed to rejection rather than a technical failure of the islet transplant. In this study, 12 of 12 dogs not given exogenous steroids were normoglycemic for at least the first 2 days following allotransplantation (Fig. 10,11). This reliable early function allows comparisons between groups even in the face of early rejection and shows the suitability of this model of allotransplantation for the comparison of various immunosuppressive protocols in islet allografting, or for assessing the results of methods of ex vivo immunological manipulation.

The cause of cardiorespiratory collapse in one animal and hypotension and respiratory collapse in another dog of this group is unknown. The effects of the vasoactive amines contained in the graft would not be exerted on the animal until after transplantation since the clamping of the splenic pedicle and short gastric vessels during transplantation effectively removes the spleen from the circulation. Once could postulate a neural (vagal) reflex in response to either mechanical forces (i.e. Sudden distention of the spleen) or local effects of chemical or hormonal products contained in the graft. A vasovagal effect does seem likely, since no further episodes were noted after the introduction of vascular loading with 1 to 1.5 litres of lactated Ringer's solution prior to transplantation.

5. Immunosuppression with Azathioprine/Prednisone

Combination immunosuppressive therapy with azathioprine and steroids, although the backbone of immunosuppression in clinical renal transplantation for nearly twenty years, has not shown dramatic results in pancreatic fragment transplantation. Bell found no delay in rejection using this combination in rodents(146). Kolb found no effect of immunosuppressive treatment with azathioprine (3 mg/kg/day) and prednisone (2 mg/kg/day) on pancreatic fragment transplantation in the dog: both control allotransplanted animals and treated animals became hyperglycemic from 1 to 3 days post transplant(159). Kretschmer achieved prolonged normoglycemia in 8 of 24 dogs allotransplanted and given azathioprine (5 mg/kg/day) and prednisone (2 mg/kg/day). All 8 subsequently died of infections thought to be secondary to excessive immunosuppression(160).

As in these previous studies we were unable to detect any significant delay in rejection or any prolongation of survival achieved by immunosuppression of allotransplanted animals with azathioprine and prednisone(Table 1). On the whole, these animals fared less well than dogs not receiving any immunosuppressive medications after allotransplant.

The danger of utilizing high dose steroids for pancreatic transplantation has been considered previously(165), and we certainly found evidence of significant diabetogenic effects. Fasting blood glucose levels on the first day post transplantation (before rejection had shown any effect in non-immunosuppressed animals) were significantly higher in azathioprine/steroid treated dogs than in autotransplanted animals, allotransplanted animals not treated with immunosuppressive medications, and allotransplanted animals given CsA (Table 1).

Significant levels of insulin ($\geq$ 2 munits/L) were present in the peripheral blood of azathioprine/prednisone treated animals for nearly identical times as nonimmunosuppressed animals (8.2 ± 0.6 vs 7.3 ± 0.8 respectively). Despite similar periods of insulin production, the treated animals "rejected", i.e. became hyperglycemic, significantly sooner than nonimmunosuppressed dogs ($P<.01$, Table 1, Fig. 12). Weight loss patterns also reflected the results of steroid stress. While untreated allotransplanted dogs lost weight at a rate of 0.24 kg/day after transplantation, azathioprine/steroid treated dogs lost weight at 1.0 kg/day initially and weight loss slowed only to 0.61 kg/day after feedings were resumed (Fig. 6, Table 3).

Just as serious were the frequent problems with sepsis and delayed wound healing seen in this group. One animal died 8 days postoperatively from a bilateral lobar pneumonia. Three animals developed wound infections and another a superficial wound dehiscence without purulence 10 days postoperatively. Wound problems were thus seen in 2/3 of these animals; such problems were exeedingly rare in other groups.

Survival figures reflected the above influences. Azathioprine/steroid treated animals had the shortest survival of all allotransplant groups, shorter even than nonimmunosuppressed animals

(Table 1, Fig. 13).

In summary, azathioprine/prednisone immunosuppression achieved no significant delay of rejection or prolongation of survival. Significant diabetogenic effects were reflected by high blood glucose levels in the early postoperative period, early "rejection" despite significant levels of circulating insulin, and accelerated weight loss. Survival was shortened, although not significantly so, and septic complications were very frequent.

6. Allotransplantation with Cyclosporin A Immunosuppression.

The pilot group of 2 dogs, given CsA orally only, rejected their grafts on days 3 and 12 and were sacrificed on days 26 and 33. Several problems were apparent with this initial group. The CsA used was approximately 2 years old and drug activity may have decreased during storage. CsA is also absorbed to only a variable degree from the gastrointestinal tract(244), the insult to the GIT resulting from total pancreatectomy could certainly result in a decrease in this fractional absorption. Without monitoring of serum levels there is no method of following the adequacy of dosages given (and the amount absorbed). Consideration of these problems led to the final protocol used in group 6b.

Previous experience with CsA in pancreatic islet or dispersed fragment transplantation is limited. Neither Garvey(232) nor Rynasiewicz(233) could significantly delay rejection of rat islets transplanted across a major histocompatability barrier. Vialettes(234) did achieve a significant prolongation of rat islet allografts and documented successive episodes of incomplete rejection before total failure of the graft. The trials of Kolb(159), DuToit(236), and of

Mahoney and Merrell(237) achieved no significant prolongation of the function of pancreatic dispersed fragment allografts in dogs despite utilizing oral doses of CsA up to 40 mg/kg/day. Williams achieved normoglycemia for over 60 days in 2 of 4 littermate DLA-MLC compatible dogs allografted and immunosuppressed with CsA 40 mg/kg/daily orally; no success was achieved with 25 mg/kg/day or with the higher dose in unrelated dogs, despite their serological (DLA) and cell mediated (MLC) assays of compatibility(238). Using cryopreserved islets for allotransplantation and oral CsA at 20 mg/kg/day Toledo-Pereyra achieved a mean survival of 35.0±31.1 days, with 4 of 7 dogs surviving over 60 days(245). He did not comment on post transplant normoglycemia.

Adequate trials of CsA with human pancreatic islet transplantation have not been carried out.

In contrast to other dog work, all 6 of our animals treated with oral/parenteral CsA were normoglycemic following transplantation. Rejection occurred from 4 to 41 days post transplant (Fig. 12); mean 19.3±5.6 days (median 15.5) (Table 1). Survival varied from 23 to 50 days (Fig. 13); mean 33.3±4.6 days (Table 1).

CsA as a single agent administered parenterally in the perioperative period and orally thereafter achieved significant delay in rejection and prolongation of survival when compared to nonimmunosuppressed dogs or those treated with azathioprine and steroids. Levels of significance for normoglycemia (Students t-test for independent groups) were: CsA treated dogs vs. nonimmunosuppressed dogs, $P < .05$, and CsA treated dogs vs azathioprine/prednisone treated dogs, $P < .02$. For survival the comparisons were: CsA vs. nonimmunosuppressed, $P < .01$, and CsA vs. azathioprine/prednisone, $P < .001$. CsA treated animals maintain a significant delay in the disappearance of insulin (< 2 mU/L) from the circulation - another marker of rejection. $P < .05$ for comparison of CsA treated dogs to both other groups.

Weight loss was similar to nonimmunosuppressed dogs over the first 4 postoperative days and considerably less than azathioprine/steroid treated animals (Table 3, Fig. 6). Once full feedings were reintroduced the rate of weight loss slowed dramatically in CsA treated animals to 0.11 kg/day from days 5 to 9 post transplant. All 5 CsA treated animals that had not rejected their grafts by the time full feedings were resumed subsequently regained some portion of their lost weight; with 2 animals gaining 0.5 kg over a 10 day period.

This evidence of sustained weight gain, normoglycemia and nondiabetic K values on IV GTT at 3 weeks post transplant (Table 2) highlight the degree to which metabolic control is normalized by allotransplantation and immunosuppression with CsA; these results being very similar to those achieved in autotransplants, although limited in duration by ultimate graft rejection.

Unlike Vialettes(234) we found no evidence of a progressive series of incomplete rejection episodes. All dogs experienced a rejection that was characterized by a rapid climb in blood glucose over a 2 to 5 day period and a fairly abrupt reduction in insulin levels either simultaneous with or 1 to 2 days following "rejection" (Table 1). Unfortunately, decreases in insulin level were found to follow and therefore not to be predictive of rejection as gauged by hyperglycemia. The production of insulin for 1 to 2 days after the onset of hyperglycemia does suggest that rejection occurs over a period of days and that with appropriate treatment of rejection episodes, graft function could be re-established. No animal reversed an episode of rejection in this study, although CsA was continued for a minimum of 5 days after the onset of hyperglycemia. Given the mode of faction of CsA[176,186], once rejection has begun, one would not expect a reversal of the process. A major problem with clinical islet transplantation will be finding a marker of rejection at an early point in its development, hyperglycemia being indicative of dysfunction or destruction of islet tissue to within 8-10% of normal islet mass (that amount estimated to be necessary to maintain normoglycemia).

Rejection occurred in all animals while serum levels of CsA were within the "therapeutic range" of 0.1 to 0.4 mg/L (Table 6). The validity of these serum ranges as guides to dosage must be questioned. Correlation between serum levels and prevention of rejection has not been well established even for human renal transplantation. Serum levels have been better correlated with toxicity, levels < 0.4 mg/L being associated with a lower incidence of side effects (notably nephrotoxicity and hepatotoxicity) at least in human studies[177].

Serum CsA levels in our animals were well above the toxic level of 0.4 mg/L, especially during the period of intravenous administration (Table 6). All levels had fallen to "therapeutic" values by 7 days post transplant. Despite high levels of CsA, clinically evident toxicity of the drug was not seen. Serum creatinine levels were elevated only intermittently and occurred in all groups with equal frequency; no evidence of nephrotoxicity was seen. A suggestion of mild hepatotoxicity was seen in CsA treated animals. Serum alkaline phosphatase levels were somewhat higher in CsA treated dogs during the first week following transplantation (Fig. 16). Differences reached statistical significance on only one day, however, and serum alkaline phosphatase values had declined to normal levels by 2 weeks postoperatively in all animals without alteration of CsA dosage. No differences were noted in serum bilirubin or SGOT between any of the groups.

These findings indicate that not only is little known about the relationship between serum levels of CsA and the suppression of allograft rejection; the correlation between serum levels and toxicity is also little understood, especially in non rodent animal models. Doses of CsA considerably above 25 mg/kg/day may be relatively safe in dogs, and may be necessary for optimal immunosuppression. Working with segmental pancreatic allografts in dogs, DuToit et al achieved prolonged (> 100 days) function in animals immunosuppressed with 40 mg/kg/day CsA, while animals receiving 25 mg/kg experienced only brief prolongation of function. The only complication reported with the higher CsA dose was a marked increased in cutaneous verucae(235).

Five of our 6 animals experienced increases in plasma glucose beginning on the second day post operatively that reached overt hyperglycemia in 4 animals (Fig. 14). One animal rejected at day 4 without returning to normal plasma glucose levels. All other animals returned to normal plasma glucose levels after periods varying from 5 to 11 days. These high blood glucose levels occurred despite normal insulin levels; in fact insulin levels were high in some animals, suggesting the presence of a diabetogenic effect of CsA at high serum levels. CsA levels were high during this period of hyperglycemia suggesing an effect of high levels of CsA on carbohydrate metabolism. A diabetogenic effect of CsA has been seen previously by Anderson in tissue culture of islets incubated with CsA(246). Gunnarson thought the effect most likely due to interference with the peripheral effects of insulin(247).

Another possible mechanism of this effect would be a disturbance of the liver's role in carbohydrate metabolism. Although no consistent effect of CsA was seen on serum SGOT, alkaline phosphatase elevations in this same period suggest a hepatotoxic effect of CsA. This correlation is obviously still speculative only.

The only major complication in the dogs treated with CsA was not due to the drug. In fact it emphasized the safety of CsA relative to previous azathioprine/steroid protocols. One of the CsA treated animals developed a complete bowel obstruction on the second day post transplantation. Laparotomy confirmed an ileoileal intussusception and resection of 40 cm of small bowel was required. Hyperglycemia ensued but after two days of insulin treatment, spontanteous normoglycemia returned and insulin treatment was stopped. The dog healed his wound rapidly, took oral food eagerly within 4 days, subsequently gained weight and had normal blood glucose and insulin levels for a considerable period before rejecting. The rapid healing of wound and GI tract and the absence of septic complications from resection of obstructed small bowel in an animal treated throughout with 25 mg/kg/day of CsA is indeed a notable departure from the frequent septic complications and poor wound healing of the azathioprine/steroid treated animals.

The relative lack of toxicity of CsA and particularly its lack of myelosuppression make it attractive to use in combination with other immunosuppressive agents. Synergistic actions of CsA in combination with both prednisone and azathioprine have been documented by previous studies(193,228,231) and deserve full evaluation in this model of pancreatic fragment transplantation.

Another aspect of CsA of which little is known, is its optimum route of administration. The most common route used is oral, however GI absorption is variable and may well be erratic in the perioperative period. Intravenous administration in our study gave much higher levels than similar doses orally, and serum levels reached very high values(Table 6). These high levels did not result in significant toxicity and threw doubt on the use of human "toxic" level figures in canine work. Interestingly, Craddock et al have found a marked improvement in survival of dogs treated with CsA 25 mg/kg/day intramuscularly following total small intestinal allotransplantation when compared at the same dosage given orally(210. There is much we do not understand about this drug, and so, still many opportunities for improvement in the results of immunosuppression using CsA alone, or in combination with other immunosuppressants.

In summary, CsA succeeded in achieving a significant delay in rejection and a significant prolongation of survival when compared to both nonimmunosuppressed allotransplanted animals and those given azathioprine and prednisone. Metabolic control achieved with allotransplantation and CsA was similar to autotransplanted animals as evidenced by normoglycemia, nondiabetic IV GTT K values, and stabilization and eventual gain of weight. This was achieved with a dose that produced minimal evidence of toxicity.

D. Studies of Cryopreservation

Cryopreservation promises distinct advantages for clinical pancreatic islet transplantation. Foremost among them at present is the possibility of long-term storage of isolated islets without the cumulative loss inherent in other storage methods. Alternative storage methods such as pulsatile perfusion, cell culture and simple hypothermic storage have all been used successfully for short-term preservation of intact pancreas and islet preparations, but all have been limited by a progressive loss of cells and viability over time.

Knight et al were the first to report successful hypothermic storage of isolated islets, but secretion of insulin was decreased by 50% within 48 hours(248). Frankel stored mouse islets at 8°C for 5 weeks and was able to retain 2/3 of insulin secretion, but only if islets were rewarmed to 37°C weekly for a brief period(249). Schulak demonstrated satisfactory function of islets stored at 4°C for up to 48 hrs(250) and most other studies have found a similar time limitation to the success of simple cold storage(251). Pulsatile perfusion of segmental pancreatic grafts has been able to reliably preseve function after transplantation only when storage was for < 48 hours(252).

Tissue culture techniques have been able to successfully preserve rodent islets for periods of over 1 week(253). Longer-term storage has not been successful, however, and the difficulties with culturing large tissue volume have prevented successful application of the techniques to large animal transplantation models.

Cryopreservation, however, can store islet tissue for prolonged periods. Rajotte and Scharp documented successful function of islets cryopreserved for 2 to 5 weeks transported between centres and then transplanted in rats(254). In vitro function of islets cryopreserved for 1 1/2 years has been documented by Bank. Long-term storage would allow time for better histocompatability matching of donor and recipient. It would also allow islet banking. Islet banking would not only allow optimal matching of donor and recipient but also accumulation of more than one suitable graft for each recipient, should multiple donors prove necessary. Storage would also give time for thorough preoperative preparation of the prospective recipient, many of whom will be poorly controlled diabetics, likely with significant associated medical problems and diabetic complications. It has been established that varying cell types and multicellular structures have different optimal cryopreservation protocols, especially cooling and thawing rates(255); toxicity from exposure to different cryoprotective agents at different concentrations and for varying exposure periods would likely also vary. This reflects the differences in size, surfact to volume ratio, membrane permeability, and tolerance of the cells to osmotic shock and ice crystal formation in these single and multicellular systems. Bank(256) and Warnock(257) have both documented evidence of selective destruction of exocrine tissue during freezing Hinshaw,et al. also noted selective destruction of exocrine cells at low temperatures(83). Should highly immunogenic cells such as passenger leucocytes and dendritic cells be subject to destruction by freezing, considerable advantage would be attained in islet allotransplantation.

Methods have been developed for cryopreservation of isolated rodent islets that yield consistent success(258). Unfortunately, direct application of these methods to canine dispersed fragments has not been successful(259). Many factors may be involved. First, species differences in cell membrane structure and cellular function may alter the kinetics of cryoprotectant permeation. Likewise, differences in optimal cooling and thawing rates may vary, as might cellular resistance to such stresses as osmotic shock and the toxic effects of Me_2SO at various temperatures(255, 254). Variations in time of exposure to collagenase and total warm ischemic time are also longer in the processing of canine tissue and may render islet tissue less able to withstand additional stresses. Canine dispersed fragments are much larger than isolated rodent islets and the need for changes in technique to accommodate this factor alone are likely.

Rajotte and Warnock achieved promising early results by increasing the temperature of Me_2SO exposure from 0°C to 25°C for the last 15 min of cryoprotectant permeation(259). Since Me_2SO permeation is temperature dependent, the higher temperature may have resulted in more complete penetration of the cryoprotectant into the relatively large pancreatic fragments. Adequate protection during the freezn thaw sequence was the final result.

Direct extension of these methods were not entirely successful. Our initial group of 8 cryopreserved dogs yielded mixed results. One animal failed completely for unknown reasons and a second exhibited only brief normoglycemia before failure. A third dog was never normoglycemic despite high peripheral insulin levels.

A technical problem with transplantation may have led to the failure of another frozen thawed graft. One cannula perforated the vein wall inside the spleen, resulting in a transplant partly by intrapulp injection rather than venous reflux. The gradual failure of the graft in this animal is similar to the pattern seen by Warnock with intrapulp transplantation of fresh islets(80).

Technical problems during the freeze/thaw sequence could be responsible for failure in two other animals. One graft was exposed to Me_2SO at 40°C for a prolonged period when a heater was inadvertently left on. The temperature dependent toxicity of Me_2SO is likely responsible for failure of this transplant. Another frozen graft was thawed at an excessively slow rate; this may have contributed to the failure of transplant in this dog.

The other 2 animals in this initial group have been long term successes. Early post-op normoglycemia was followed by a short period (1 to 4 days) of hyperglycemia, then by normoglycemia that has persisted for over 12 months. K values have remained over 1%/minute for 12 months in both, 1 animal subsequently became diabetic and was sacrificed, the other was sacrificed during metabolic testing 19 months post transplant.

Technique was changed only marginally in the last group of 4 dogs transplanted with FT islets, all of which were successes. Transfer from Erlenmeyer flask to cryo test tube was performed on ice after cooling the Erlenmeyer in ice for a 5 min. period. The success of these final 4 dogs is more likely due to a "practice effect" than to this minimal change in technique.

Another problem was surmounted by an alteration in equipment. Freezing of dispersed fragments from an entire canine or human pancreas by our current techniques requires over 20 test tubes of cells. Nucleation by ice crystal addition is tedious with such numbers and exposes the cells to contamination. Nucleation by external contact of the glass "cryo-tube" with a metal rod cooled in liquid nitrogen eliminated this chance of contamination but remained inefficient. Installation of an FTS pump to circulate liquid nitrogen through a stainless steel tube which was molded to run near the edge of the ethanol bath created a nucleation system that could handle large numbers of tubes within a few minutes. This more efficient system also resulted in more satisfactory nucleation, all cells being frozen in suspension, with minimal settling seen.

The development of aluminum holders in the evacuated Dewar flasks also aided in streamlining the procedure by speeding the transfer of test tubes from the ethanol bath for final controlled cooling. This rapid transfer prevented warming of tubes by ambient air during transfer and eliminated problems of inequality of temperatue in the test tubes in the ethanol bath and the ethanol in the cooling Dewar's at time of transfer.

Results in the 4 dogs of group 7B were a dramatic improvement. All were normoglycemic in the early post-operative period, only one experienced any period of hyperglycemia (Fig. 4) and all were normoglycemic for over 15 months post-transplant. K values on glucose tolerance testing have been superior to control fresh autografts at all periods up to 12 months, although differences have not reached statistical significant (Table 2). Similarly, peripheral insulin responses to glucose infusion in the frozen/thawed animals have been consistenly better than in freshly autotransplanted animals (Fig. 9, Table 5) though differences again are not significant at the 5% confidence level. There has been little evidence of fatigue of islets transplanted after cryopreservation over the 15 to 18 months of follow-up.

Weight loss has also been controlled in these frozen thawed animals Fig. 7, Table 7). Two dogs lost weight for the first 3 months after transplant then stabilized. The other 2 animals lost weight for a period of 1 month only. Both subsequently gained weight and indeed weighed more at sacrifice than they did preoperatively. The longer period of initial weight loss in these animals as compared to freshly autotransplanted dogs may represent the time period required for correction of freeze/thaw induced injury. It is tempting to speculate that the long-term weight gain over pre-operative levels in 50% of frozen/thawed dogs is due to superior long-term performance of the cryopreserved dispersed fragment gemische, as is suggested by the higher K values achieved on glucose tolerance testing and the greater level of insulin response to glucose infusion.

Dogs transplanted with frozen/thawed islets have thus experienced marginally better metabolic control post-operatively than animals receiving fresh islets. Several factors may be responsible. The animals used for cryopreservation studies had both a higher average pre-operative K value and a slightly higher average volume yield of tissue from the isolation procedure, although neither difference was statistically significant.

The differences may also be explainable by considering possible consequences of the freeze/thaw process. In the islet the glucagon producing alpha cells lie periphrally. This position could leave them more open to damage; it could also afford them better potection due to more complete Me_2SO equilibration. Previous histological studies have not indicated preferential alpha cell damage(80).

It is well established that varying cell types require different cryopreservation techniques(255,254). Endocrine cells have previously been thought to be more resistant to cold injury and freeze/thaw injury than exocrine cells(83,256). Transplantation of an unpurified pancreatic fragment gemische leaves endocrine cells in immediate proximity to exocrine cells in the host tissues. Enzyme products of the exocrine tissue could conceivably damage or destroy a significant amount of islet tissue. Preferential destruction of exocrine cells during the freeze/thaw process may protect the islets from this damage and so be responsible for the superior long-term metabolic performance seen in this study.

An extension of this line of thought reflects on immunoalteration. Should cryopreservation also be capable of selective destruction of passenger leucocytes, dendritic cells, and other highly immunogenic non endocrine contaminants, another major advantage would be gained in clinical allotransplantation by the incorporation of cryopreservation techniques. Our final experimental group attempted to test this postulate.

Unfortunately, the combined stresses of collagenase digestion, cryopreservation and allotransplantation appeared to be additive. Three of six dogs were never normoglycemic, despite significant insulin levels for 5 or more days in all (Table 1). Although initial post-operative blood glucose levels were under 200 mg/dl in all 3, rejection ultimately occurred before repair of the damaging effects of the freeze/thaw sequence was complete enough to result in normal blood glucose levels. The definition of engraftment and subsequent rejection employed by this study eliminates them from consideration. Comparison between freshly allografted animals and the remaining 3 frozen/thawed allografted animals did not reveal significant differences in either time to rejection or survival.

Promising indications were seen, however, in this small series. One animal did not reject his graft until 12 days following transplant, a considerable period of graft function. Another, although becoming hyperglycemic on day 5 post transplant, continued to show substantial levels of insulin in peripheral serum for over 25 days, and survived for 58 days, considerably longer than any non frozen allografted dog. Further examination of this exciting aspect of cryopreservation is needed.

VII CONCLUSIONS

1. These studies verify the results of Warnock with this model of pancreatic dispersed fragment autotransplantation in the dog. The model is reliable and achieves a level of metabolic control, of the severe form of diabetes mellitus that results from total pancreatectomy, that is equal or superior to that of any other published method.

2. This metabolic control is enduring, as evidenced by the failure of only 1 of 5 autografts over a period of follow up of greater than 18 months (a second animal died at 4 months due to a bowel obstruction).

3. This pancreatic dispersed fragment preparation is not satisfactory for intraportal infusion in its present form.

4. This method of pancreatic dispersed fragment preparation and transplantation by splenic venous reflux is satisfactory for allotransplantation. The absence of any sustained period of hyperglycemia post-transplantation makes this model suitable for comparison of varying methods of recipient immunosuppression, of ex vivo immunological manipulation of tissue for transplantation, or of other methods to achieve delay or prevention of allograft rejection.

5. Recipient immunosuppression with azathioprine 3 mg/kg/day and prednisone 2 mg/kg/day was not able to achieve any delay in rejection of the implanted islets or any prolongation of survival over non-immunosuppressed allograft recipients. The transplanted islets tolerate the diabetogenic stress provided by this dose of prednisone poorly as evidenced by earlier onset of hyperglycemia and earlier death when compared to the nonimmunosuppressed allograft recipients.

6. Cyclosporin A 25mg/kg/day, orally, combined with parenteral administration in the perioperative period, is capable of significant delay in rejection of transplanted pancreatic dispersed fragments as well as prolongation of survival of recipient animals when compared to both nonimmunosuppressed allograft recipients or those treated with azathioprine and prednisone.

7. With this model of pancreatic dispersed fragment transplantation, and utilizing immunosuppression with either Cyclosporin A or azathioprine and prednisone, no evidence of a progressive series of incomplete rejection episodes was seen. All rejection episodes proceeded to complete loss of islet graft function inexorably within 1 to 4 days of the first indication of hyperglycemia. The presence of insulin in the peripheral blood after the onset of hyperglycemia does suggest the possibility of reversability of the rejection process, that is, it seems that immune destruction of all the engrafted pancreatic islets does not occur simultaneously.

8. Cyclosporin A at the "therapeutic levels" of 0.1 to 0.4 mg/L that are often utilized for human cadaver kidney transplantation is not capable of indefinite prevention of rejection of allotransplanted pancreatic dispersed fragments in the dog. This factor when combined with the decline in serum Cyclosporin A, levels observed in these pancreatectomized dogs suggests that either an increase in drug dosage will be required in the later postopeative period to prevent rejection, or that a combination of immunosuppressive medications will be required to prevent rejection of allotransplanted canine pancreatic dispersed fragments.

9. No evidence of toxicity to renal or hepatic function was seen in this study with serum levels of Cyclosporin A that were well above 0.4 mg/L in the early postoperative period. This lack of renal toxicity of CsA in dogs has been noted by many other investigators.

10. The metabolic control achieved in allotransplanted dogs successfully immunosuppressed with Cyclosporin A 25 mg/kg/day is similar to that achieved by autotransplantation of pancreatic dispersed fragments. Evidence was seen for an adverse effect of Cyclosporin A on carbohydrate metabolism in the early post-transplant period when serum CsA levels were high.

11. Cryopreserved canine dispersed pancreatic fragments (slow cooled to -75° after permeation by Me_2SO, stored in liquid N_2, then slow

thawed and cleared of Me_2SO by sucrose addition) are capable of successful long term amelioration of diabetes mellitus after autotransplantation. A relatively reliable method was developed that acheived metabolic control at least as good as acheived by dispersed pancreatic fragments autotransplanted immediatley after isolation.

12. No significant delay of rejection, nor prolongation of survival was seen when canine dispersed pancreatic fragments were cryopreserved prior to allotransplantation.

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